

Contaminated blood continues to boil

Last week's complaint by 97 mostly French physicians draws attention yet again to the need for a wider inquiry into the origins of the contaminated blood scandal.

THE French scientific community has been astonishingly laggard in reacting to the imprisonment of the two people sent to jail last year for their part in the scandalous use of blood contaminated with HIV for the treatment of French haemophiliacs. The letter now sent to President François Mitterrand (see page 312), which argues that the sentences being served by Dr Michel Garretta and Professor Jean-Pierre Allain are unjust (as are the convictions, without sentence, of two others), may bring some comfort to the two scientists now in prison, but they are unlikely to carry much weight with the president. For one thing, Mitterrand will be able to say that it would be improper for a politician to intervene in the judicial process. For another, he has no incentive to reopen an inquiry into the conduct of his own government in the summer of 1984, when the National Transfusion Service continued to supply contaminated blood products "while stocks last", in Garretta's infamous phrase.

There is no doubt that the sentences handed down last summer, at the end of what was essentially a second trial of the case against the four defendants, are unjust in an important sense. What went wrong in France in the early summer of 1985 was that the government decided to wait until the autumn, when a French rather than a US blood test would become available, before instituting the routine screening of blood for transfusion and the manufacture of blood products. How and why the decision to delay was taken has not been fully explored, but there is no doubt that the result was to confirm the newly privatized transfusion service in its (or Garretta's) complacent policy of wringing the full economic value from its existing blood-bank, much of which was already contaminated with HIV. Three years ago, Mitterrand himself appeared to accept that responsibility might lie more widely than with the National Transfusion Service, but his declared intention to set up a parliamentary commission to investigate the role of the prime minister (M. Laurent Fabius) among others was discovered to be out of time.

Those now in jail, or serving suspended sentences, are therefore scapegoats in some sense or other, and will rightly be so regarded at least until the government's own role in the affair has been fully investigated. Although the transfusion service had ceased formally to be a part of the public service (legally, it had become a public foundation), it remained subject to government supervision. Moreover, the government committees concerned knew of the dangers of contaminated blood — one of the charges against Dr Jacques Roux, then a senior official at the Ministry of Public Health,

is that he had been so informed in writing by his co-defendant Allain. In many ways, the two contaminated blood trials thus have much in common with the infamous (but aborted) Matrix-Churchill case in Britain, when officials of a company were prosecuted for selling arms illegally to Iraq despite the British government's knowledge that they had been acting in accordance with a secretly changed policy. The pity, in France, is that the law has been allowed to run its full course.

The treatment of Allain is especially hard. He argued early and often that it would be dangerous to continue using contaminated blood. In the eyes of the courts, his guilt is to have known of the danger when the policy of the transfusion service remained unchanged. But what, in such circumstances, is a public official to do? To broadcast his disquiet to the public, or to attempt to work within the bureaucracy that employs him? By their judgement, the French courts have placed onerous responsibilities on all potential whistleblowers, but have offered them no protection. The French government will find that an awkward precedent.

None of this diminishes the difficulty in which the government now finds itself. Although the victims of the contaminated blood and their families have been compensated financially, mere money has evidently not assuaged their outrage. Moreover, there is no doubt that scientists in public positions who, by neglect or mistaken advice, are responsible for public catastrophes, should be as accountable for the consequences as are other kinds of professional people. The danger now is that the case of the contaminated blood will permanently sour relations between the profession and the public, from which both will suffer. □

The plutonium regime

Something must be done to safeguard plutonium internationally and to prevent the threat of its misuse.

A MERE four kilograms of weapons-grade plutonium with a volume of less than 250 cubic centimetres is enough to make a nuclear weapon. That is an important part of the incentive for the study over the past two years (see page 307) by the US National Academy of Sciences, and published on 31 January (*Management and Disposition of Excess Weapons Plutonium*). Given that the theft of such small quantities of a metal is physically within the grasp of children, the



safeguarding of plutonium stocks is a vital issue.

There are two other reasons why the study, commissioned by the US National Security Agency, is both necessary and timely. First, the break-up of the Soviet Union has created huge uncertainty about the disposition in the successor states not just of plutonium but of fissile material generally. Second, it is now only a year before the future of the Nuclear Non-Proliferation Treaty (NPT) has to be determined; the reputations of those engaged on the academy's study makes it likely that their document will have an important influence on the negotiations at Geneva. That, at least, must be the hope.

Several problems lie ahead, of which the most immediate is the prospect that something will have to be done with the fissile material taken out of nuclear warheads due for dismantling under the Start-I and Start-II arms control agreements, neither of which is yet in effect. Weapons-grade uranium (enriched to 90 per cent or more in ^{235}U), is no great problem, the committee says; simply dilute it with ^{238}U and use it as reactor fuel. That is the spirit in which the United States has offered to buy fissile uranium from Russian warheads, selling it on to reactor operators as opportunities arise. It is as well to remember that even this solution requires that the successor states, and especially the Ukraine, should continue to comply with the agreements they have made. But there is at least a chance of that.

Plutonium is a much more serious problem, partly because civil reactors are likely to be producing more than 50 tonnes of it a year for the remainder of this decade, and also because there is no easy way of disposing of it. Some reactor operators in Europe and Japan are using plutonium and uranium oxides mixed together in so-called 'MOX' fuel, but the cost is higher (because of the extra radiological and security safeguards needed for plutonium). That, no doubt, is why the United States has not, or not yet, offered to take Russia's excess plutonium off its hands. (It is interesting, but a little depressing, that the committee reckons that regulatory approval for steps of this kind will be hard to come by in the United States.) It is also relevant that France, Japan and Russia have set their hearts on developing a viable fast reactor. But what else is to be done?

The committee argues cogently for a bilateral agreement between the United States and Russia in the first instance, and then for the engagement of the International Atomic Energy Agency (IAEA) in an international effort for the systematic storage and safeguarding of plutonium stocks. It probably makes good sense to see whether a practical arrangement can be worked out between the two governments likely to be the chief sources of excess plutonium in the immediate future.

But bilateral agreements, even if decked out with arrangements for mutual inspection at will, cannot readily be generalized to the multilateral arrangements there will eventually have to be to cover the diversity of plutonium producers a century from now. Why not go the whole way and transfer legal ownership of plutonium and the physical management of stocks thereof to the IAEA, with the understanding that this dangerous material would be released to its

original owners only when there is proof that it will be used in an approved fashion, perhaps as reactor fuel? The NPT is in need of what Mr Mikhail Gorbachev used to call "new thinking"; an international system of bonded warehouses for plutonium might be just what is needed. □

Agenda for EU research

Britain's House of Lords has a succinct comment on how Brussels runs research.

ONE of the few elements of continuity in the administration of British science and technology is the House of Lords Select Committee on Science and Technology, which from time to time answers Lenin's question "What then should be done?" in an appropriately magisterial way. Its best-known pronouncement is its plea in 1981 for a science minister, now granted after a decade's delay. Last November, the committee interviewed the Commissioner for Research at Brussels, Professor Antonio Ruberti, about the European Union's research programme. Its reflections on the meeting were published earlier this week as a transcript of the conversation and a letter to Mr William Waldegrave. The committee could do worse than sink its teeth into the issues that arose last November.

First, there is the question of rigidity. The standard way of working at Brussels is to struggle for a five-year research programme (called a "framework" programme) and then to spend the money (four per cent of Europe's total research budget) on predesignated schemes. But is not a programme set in concrete so far ahead almost certain to be ill-matched to the need? What the committee learned is what outsiders have always known, that there is little room for flexibility. More disturbing, the administration of the budget is likely to be more and not less rigid as a consequence of the Maastricht Treaty, which requires that all European institutions (including the parliament) should concur on spending plans.

Second, there is a matter of transparency. How is the framework budget constructed? Plainly, and properly, the Select Committee took fright at Ruberti's references to "considerations of continuity" as well as to "pressures from industrial and scientific concerns" among the influences moulding the research budget, and says in its letter to Waldegrave that it would like to see more openness in the determination of what Brussels wants to spend. It could have gone further and asked for an annual report on the EU's research programme and even for an annual budget cycle.

Finally, there is the question of what kinds of projects the EU should be supporting. The Select Committee urged on Ruberti the notion that the EU should be supporting projects intrinsically too large for member states, which would have the advantage of conformity with the principle of subsidiarity (that Brussels should do what member states individually cannot). The case for following such a course is intellectually irresistible, while Brussels is now better placed than for a decade to pursue it. That way, it would make even better use of its four per cent, and win friends as well. □

Public protests spark 'witch-hunt' fears over radiation experiments

Washington. Scientists responsible for carrying out radiation experiments on human subjects in the 1960s should face criminal investigation, a critic of the experiments has told a US congressional committee. And government officials who allegedly concealed some of the experiments could also face the wrath of Congress.

Both the Clinton administration and Congress are seeking to extract maximum political capital from the outcry that has surrounded recent revelations about the experiments. But both are also playing safe by pinning the blame on dead scientists and anonymous officials working under previous administrations.

As public interest grows, however, this approach may prove unsustainable. Many of the individuals involved are still alive, and could face prosecution over their involvement in the research, which was carried out to investigate the effects of radiation on the human body.

Addressing the House of Representatives' energy and power subcommittee last week, David Egilman, a physician from Brown University, Rhode Island, called for the criminal investigation of scientists involved.

Egilman says he plans to take evidence on experiments conducted between 1961 and 1972 at the University of Cincinnati to

the city's prosecutor. He says the research resulted in the deaths of at least eight (and perhaps more than 20) of its 88 subjects, and will ask the prosecutor to indict surviving members of the research team on charges of murder.

At the same subcommittee hearing, Hazel O'Leary, the Secretary of Energy, promised to give Congressman Edward Markey (Democrat, Massachusetts) the names of department officials who Markey claims concealed information from him during a 1985 investigation into the issue. O'Leary later denied that this would start a witch-hunt within her department: "I'm not after punishment for punishment's sake," she says.



O'Leary: naming names.

Meanwhile the official inquiry into all radiation experiments conducted in the United States since the Second World War, now being supervised by an advisory committee set up last week by Clinton's executive order, is broadening its scope.

Initially, the Department of Health and Human Services (HHS), which runs most

biomedical research in the United States through the National Institutes of Health (NIH) and other agencies, wanted to be kept out of the inquiry, apart from offering advice to the Energy Department and other agencies under investigation.

But last week it changed tack and started to set up a working group to sift through all relevant research carried out by the department between 1945 and 1975. "We don't know whether there were radiation experiments done by NIH or not," says Avis LaVelle, a spokeswoman for the health department.

The decision by HHS and other departments to be open about radiation testing has been motivated by the the popularity the administration has found on this issue since O'Leary stumbled across it last month. According to a survey in the *Wall Street Journal*, 63 per cent of Americans consider it "an important issue" compared with 41 per cent for the so-called Whitewater financial scandal dogging the president, and just 29 per cent for allegations about his sex life.

But what is good for the president may be bad for science's public image. Charles Vest, the president of Massachusetts Institute of Technology (MIT), appeared to acknowledge this when, in an unprecedented public apology for MIT's failure to obtain the fully informed consent of subjects for some experiments in the 1950s, he sought to reassure the public that safeguards have improved since then.

"Medicine has recognized past errors, studied them and really worked to put its house in order," says Dr Mark Siegler, a professor of medicine and director of the Center for Clinical Medical Ethics at the University of Chicago. The conduct of research into human subjects "has evolved to a stage where subjects are given enormous protection," he says.

But Sheldon Krinsky of Tufts University, a former chairman of the scientific freedom and responsibility committee at the American Association for the Advancement of Science (AAAS), fears that public trust in science will be undermined.

Krinsky says the radiation work was "unconscionable" in the face of clear guidelines widely circulated in the wake of Nazi war atrocities. "I'm not sure how deep the mistrust will be, and how to win it back," he says. "But the first thing to do is to set the record straight."

The extent to which rules governing the conduct of research have improved will be the subject of further hearings this week by the Senate Governmental Affairs Committee, chaired by John Glenn (Democrat, Ohio).

Colin Macilwain

Nerve gas volunteers demand follow-up study

London. A group of former servicemen who have taken part in experiments at the Chemical and Biological Defence Establishment at Porton Down since the Second World War is planning to press the government to release detailed information about the nature of the tests in which they were involved.

The servicemen are also planning to push for a long-term follow-up study on volunteers who took part in the tests, including those involving the use of mustard and nerve gas. They want to know whether any of them are suffering from health problems that might have resulted from the tests — and, if so, plan to demand compensation from the Ministry of Defence (MoD).

Up to now, the ministry has resisted demands both to open up their records to public scrutiny and to carry out long-term studies of volunteers. Officials have argued that a long-term follow-up study might spread unnecessary alarm among volunteers who remain healthy.

But Mike Roche, a former serviceman who is coordinating the new pressure group, and who took four years to persuade the MoD to release details to his doctor of the

tests he was involved in, says that the recent decision by the US administration to offer compensation to volunteers should now be repeated in Britain.

Roche has written to almost a hundred regional British newspapers asking for individuals involved in the tests to get in touch with him about putting pressure on the government.

He says that he has received many replies from individuals who want to join his campaign (Roche's address is 14 Corporation Road, Rochdale, Lancashire OL11 4EU).

Others are demanding an inquiry into experiments that took place at St Thomas's Hospital in London in the early 1960s in which patients suffering from terminal cancers were deliberately infected with a form of encephalitis from which several of them subsequently died.

In reply to a parliamentary question earlier this month, Graham Pearson, the current director of Porton Down, revealed that tests involving service volunteers are still being carried out at the research establishment, some involving exposure to the nerve gas, sarin. □

Petition prompts backlash against scientists

Paris. The front pages of every French newspaper were covered last week by an outpouring of public anger against a petition from 98 researchers, physicians and public health workers asking President François Mitterrand to pardon four doctors convicted in the recent HIV-contaminated blood affair (see *Nature* 367, 206; 1994).

This protest against what is being widely perceived as a closing of ranks by scientists and physicians has revealed how much damage the affair has inflicted on public trust in the biomedical community. Maxine Schwartz, director of the Pasteur Institute, describes the public's reaction as a "crystallization of the problem between science and society".

Haemophiliac groups condemned the petition as "shocking", and the Professional Association of Magistrates said it was "an insult to the victims." Michel Rocard, leader

of the Socialist party said he was "astounded and worried" by the scientists' action.

A co-discoverer of HIV, Françoise Barré-Sinoussi, was one of the two main signatories of the letter. She has argued that the version of events in 1984–85 given by the media and during the trial "bore no relation" to the reality facing scientists at the time.

But two of the other co-discoverers — Jean-Claude Chermann and Luc Montagnier — refused to sign the petition. Chermann said that "a scientist must be responsible by his acts and words", describing Jean-Pierre Allain, the former official at the National Blood Transfusion Centre who was given a four-year jail sentence (two of which were suspended) as a scientist who "fully knew" that the products were contaminated.

The newspaper *Le Figaro* pointed out that "the central core" of the signatories — about 20 in number — work or have worked at the Pasteur Institute. It suggested that they were "closely or distantly" involved in the commercialization of the controversial French AIDS test.

It went on to identify the second major group of signatories as former officials at the French National Blood Transfusion Centre, and described foreign signatories as "friends of Allain".

Jean-Claude Gluckmann, the second main signatory of the petition, admits that the initiative backfired. But he defends it as well-intentioned. "We hoped that people would re-examine the problem more calmly than during the trial and realize that things were not so simple. We got the opposite effect to what we wanted. The petition was not against the victims, but we cannot repair an ordeal by an injustice," he says.

Jean-Baptiste Brunet, the director of the European AIDS Centre and a signatory to the petition, says Gluckmann made a mistake by sending it to the news agency Agence France Presse. "It was like a fire that he could not stop, it exploded in his hands," says Brunet. "You have to be prudent; a lot of people's lives are involved in this story."

Brunet says that he signed the letter to protest at the way in which the four who were convicted had been made scapegoats. "France has tried to avoid the fact that the responsibility was wider," he says, claiming that Allain's conviction in particular was unjust. "They needed [to charge] a doctor, and they found one."

Brunet, who himself now faces charges of "poisoning" (see below), claims that the French press, government and public has turned a blind eye to many questions unanswered by the blood trial, which concerned only contamination of clotting factors.

France was not the slowest country to introduce measures to prevent contamination of blood by HIV. But there is evidence that in France the risks were known and deliberately ignored for industrial and commercial reasons.

First, there is the question of contamination by whole blood (see *Nature* 364, 260; 1993). Government officials are alleged to have delayed approval of a US AIDS test for five months in 1995 to allow a French test to be released first (see *Nature* 353, 197; 1991). But, despite supporting evidence, the claims have never been closely examined.

Second, health officials collected blood from high-risk populations (drug addicts) in prisons until 1986, in spite of being aware of the risks. The impact of this neglect can be estimated from the fact that more than 1,300 people in France were contaminated by HIV through whole-blood transfusions, whereas fewer than a hundred were infected in the United Kingdom, where obligatory screening for HIV was introduced two months later than in France.

Whatever the outcome of the latest developments, the public's perception that a reluctance in the scientific community to take up the haemophiliacs' cause was tantamount to complicity has been further reinforced by the petitions. And, as the newspaper *Libération* put it, referring to the recent approval by the Senate of new bioethics laws: "Placing the bar of scientific responsibility too low is an invitation to others to set it higher."

Declan Butler

New charges in HIV blood affair

Paris. Last week's petition for an amnesty for those convicted in the HIV-contaminated blood affair in the mid-1980s (see above) coincided with a new wave of legal proceedings against individuals involved.

A 19-year-old haemophiliac, Ludovic Bouchet, has brought poisoning charges against three former ministers: Laurent Fabius, then prime minister, Georgina Dufoix, minister of social affairs, and Edmond Hervé, secretary of state for health.

Related charges are also being brought against François Gros, an adviser to Fabius and former director of the Pasteur Institute; Charles-Henri Filippi, director of Dufoix's cabinet; Gaston Rimareix, the director of Hervé's cabinet; Claude Weissenberg, an adviser to Hervé; Bahman Habibi, director of France's national blood transfusion centre at the Saint Antoine Hospital in Paris; and Jean-Baptiste Brunet, director of the European AIDS centre.

The charges have coincided with the introduction of a reform of the High Court of Justice that allows the prosecution of former ministers (see *Nature* 364, 269; 1993).

"I have done my best over the past 13 years to fight AIDS," says Brunet. "It's horrible for me to be charged with taking part in the epidemic." He says the charges against him are based on memoranda written to the government alerting them to the risk from contaminated blood, which are now being interpreted as indicators of his guilt, as he knew what was happening. "Of course I knew," says Brunet. "That's why I wrote the letters."

D. B.

French academy backs the use of English

Paris. The French Academy of Sciences last week defended the right of French researchers to publish and present their results in English, and said it opposed any legislation that would restrict this right.

A bill on the use of the French language is due to be introduced into the National Assembly in spring, and is expected to require scientists to use the French language at conferences both inside and outside France, as well as introducing other curbs on the use of English.

The academy's decision to go public with its opposition is thought to have been prompted by the lack of response from the government to similar recommendations which academy officials made privately last September.

In its statement, the academy also encourages researchers to publish books and articles in French, to meet the needs of students and others interested in science, and promote scientific culture in the French-speaking world.

D. B.

Japan prepares to mark its independence in space ...

Tokyo. Japan's space programme will enter a new era next week with the launch on Tuesday (1 February) of the first H-II rocket. This is a vehicle made entirely in Japan that will be able to compete with US and European rockets in terms of payload capability, and can be used to launch large Earth observation satellites.

The development of the H-II has been plagued with difficulties. Next week's launch is two years behind schedule because of setbacks in development of the liquid-fuel combustion engine in the first of its two

stages; the engine burst into flames on several occasions during testing, and also killed an engineer during pressure tests of a component (see *Nature* 352; 651; 1991).

But if the launch is successful, Japan will have broken free of its dependency on US launcher technology, and will have a powerful launch vehicle capable of placing a two-tonne satellite in geostationary orbit.

Japan is unusual in having two space organizations: the Institute of Space and Astronautical Science, operating under the Ministry of Education that launches small

... as France and Germany end ESA dispute

Paris. Determined to present a united face to its non-European partners in the international space station, the European Space Agency (ESA) last week ended a dispute between Germany and France over funding for their favoured manned space programmes by approving both programmes within a new single strategy.

A joint Franco-German team will now manage both the German-led Columbus programme — which includes the Attached Pressurized Module (APM) for the international space station Alpha, and flights by Europeans on the Russian Mir station — and the French-led Manned Space Transportation Systems (MSTP) programme.

The latter is a revised version of the abandoned Hermes shuttle programme: it now includes the development of the launcher Ariane V and studies on an Apollo-like capsule to be mounted on the nose of the launcher, a Euro-Russian space suit and a Dutch-built robotic arm.

The agreement breaks the deadlock over ESA's 1994-95 budget for manned space activities caused by disagreement between France and Germany over their contributions to each other's programmes. ESA's manned space programme will now get ECU477.4 billion (US\$540 million) over the next two years, corresponding to ECU270.4 billion for Columbus and ECU207 billion for MSTP.

Formal approval of the budget will depend, however, on the successful outcome of a separate dispute over ESA's financial mechanisms at a meeting of council next Monday (31 January). Both Italy and Spain will seek redress for increases in their subscription fees due to a recent decline in the value of their respective currencies.

The endorsement of a common strategy by ESA's member states — including Italy and Spain, which some had feared might withdraw support — will alleviate a period of uncertainty over Europe's space station

plans, says Jean-Daniel Levy, the director-general of the French space agency CNES.

Levy says that, with the United States and Russia now committed to an international space station, "we have a much clearer idea of where we going than last year". Jim Thomas, deputy director of the British National Space Centre, agrees that the situation has stabilized. But he points out that ESA's plans remain at the mercy of uncertainty in the United States and Russia over the shape of the space station.

Some still fear that if the station falls through, ESA will be left with a costly programme it does not need. ESA argues that its revised plans could enable it to build its own orbiting laboratory; but many believe this argument is more a negotiating weapon than a serious proposition.

Furthermore, ESA's contributions to the space station will hang in the balance until formally endorsed at next year's meeting of the space ministers of its member states. The economic recession in Europe, and falling interest in space in many European countries, could lead to yet another round of cuts in the manned space programme.

ESA also faces a series of medium-term threats. The agency already accounts for less than half of Europe's space activities; indeed its budget is less than that of the French space agency CNES. Mark Gidget of the Paris-based space consultancy Euroconsult says that most advanced European countries have expressed a preference for working alone or directly with the United States or Russia. If interest drops in the agency's big programmes, "its *raison d'être* will disappear", he says.

The biggest threat to ESA in the long term is European political union, says Gidget. The Maastricht treaty has extended the European Commission's role in science and defence, and observers say it is bent on gaining control of space programmes.

Declan Butler



Japan's Orbital Re-entry Experiment Vehicle (OREX) will be one of H-II's first payloads.

scientific payloads with Japanese-developed solid fuel rockets; and the National Space Development Agency (NASDA), affiliated to the Science and Technology Agency, which launches larger Earth observation and communications satellites and has until now been using US technology in its launch vehicles.

The H-II will be used to launch at least two large Earth observation satellites this decade that will carry US and European as well as Japanese sensors. One, the Advanced Earth Observing Satellite (ADEOS), is scheduled for launch in 1996, and will have ozone sensors and other equipment for monitoring the Earth's environment.

The Tropical Rainfall Monitoring Mission, a joint project with the US National Aeronautics and Space Administration, will be launched a year later to observe clouds and rainfall in tropical regions. A second ADEOS satellite is tentatively scheduled for launch at the end of the decade.

Although Japan can now compete with US and European rockets such as Ariane in terms of payload capability, it remains to be seen if the H-II can break into the lucrative commercial market for the launch of communications satellites.

Rocket Systems Corporation, a consortium of 75 Japanese companies, has been set up to bid for orders for launches from organizations such as the International Telecommunications Satellite Organization (see *Nature* 352, 651; 1991). But the H-II is at present limited to two short launch windows a year under an agreement with local fishermen at NASDA's launch site in Tanegashima island. Furthermore, the price of H-II launches is higher than that of competitors.

Early in the next decade, NASDA is planning to use the H-II for launches of an unmanned space shuttle called HOPE (H-II Orbiting Plane) now under development. This will be capable of carrying payloads of several tonnes into low Earth orbit, and may be used to transport equipment to and from the US Space Station Freedom.

The H-II launch next week will carry an orbital re-entry test vehicle, resembling the nose cone of HOPE. It will be placed in orbit, return to Earth and splash down by parachute in the Pacific Ocean a few hours after launch.

David Swinbanks

Industrial research in crisis in east Germany

Bonn. Despite substantial government support, industrial research in east Germany is on the verge of collapse. Figures published earlier this month reveal an 80 per cent drop in the number of research staff in industry over the past three years.

Germany's research minister, Paul Krüger, says the government will continue its high level of direct support for such research. But he is also planning a programme to help establish high-technology industries in the new *Länder* as a way of encouraging more industrially relevant research and development.

The new figures, published by the Public Society for Science Statistics, are a major disappointment for the government, which has spent DM1.62 billion (US\$950 million) over the past three years on supporting the industrial research base in east Germany.

When the Berlin Wall was pulled down

in 1989, about 86,000 people were employed in research departments in east German industry. This was judged to be an overcapacity of around 16,000 by western



Krüger: admits errors.

German standards. Since then, the numbers have fallen rapidly. By 1991, only 34,600 were employed in industrial research and development; the predicted figure for 1994 is 16,000.

The fall partly reflects the declining fortunes of industry in the new *Länder*, following the collapse of its East European markets and the recession in the West. But this is not the whole story. The companies

that have survived employ relatively few research staff; only 2 per cent of industrial employees work in research and development in the new *Länder*, compared to 4 per cent in the rest of Germany.

Krüger, who himself worked as a research group leader for a mechanical engineering firm in the former East Germany, is concerned at the extent of this decline.

"We give proportionately eight times more money to the new *Länder* for industrial research compared with the old *Länder*, and this is still not enough," he says. But he stresses that there is a limit to the amount of support the government is prepared to provide to an industry that is less successful than that in west Germany in delivering new products to the marketplace.

Krüger admits that different policies might have prevented the collapse of industrial research during the early days of reunification. During the restructuring period, for example, Treuhand — east Germany's privatization agency — encouraged the dismantling of the large conglomerates into smaller units, to prepare them for privatization.

At that time, most research departments were separated from their parent companies, and became independent contract research companies. But many now recognize this to have been a mistake. At least a third have not survived, and most of those that remain are heavily dependent on government subsidies. "At the time, researchers thought they would have a better future if they were independent," says Krüger. "They overestimated their potential."

Krüger says he regrets that Treuhand did nothing to discourage researchers from setting up independently, and that not enough money was made available during the transition period. He argues that Western standards were imposed too rapidly onto the east, and that too many research groups were left to sink or swim. "I would have tried to keep the research resources that were available, using government funds, to make the transition [to a capitalist economy] easier."

Given that products made in east Germany are generally uncompetitive on the world market, Krüger, while maintaining the many different programmes for support of industrial research, is also working with Treuhand and the Ministry of Industry on a new programme to encourage the development of more innovative, high-technology products in the east.

Details of the programme have not yet been finalized. But DM150 million has been allocated to it by the government in 1994. This money comes from funds belonging to the communist party of the former East German republic that are being used to rebuild the industrial base of the new *Länder*.

Alison Abbott

Differing views on technology transfer

Bonn. A united Germany's first parliamentary debate on science and technology, which took place in Bonn earlier this month, underlined sharp differences between the political parties about how to improve the interaction between research and industry.

The debate was prompted by increasing political and economic pressure to improve efficiency of technology transfer. Germany has a strong economy and a well-financed and effective fundamental research base. But the two sectors do not work well together. The rate of increase in patent applications is falling, and Germany competes poorly with other countries in many high-technology areas.

In October 1992, a report commissioned by the state of Baden-Württemberg recommended setting up a federal technology council. This five-member council, meeting under the aegis of the federal president, would make recommendations on the financing and organization of research judged necessary for long-term economic growth.

The report was put together by a committee dominated by a number of prominent German industrialists, but also including academics and politicians, and has stirred considerable controversy in political circles. The Christian Democrat party (CDU) has been divided in its response, while the Free Democrats, partners with the CDU in the ruling coalition government, oppose the recommendations, fearing they will encourage state interference in issues that should be left to industry.

In contrast, the opposition Social Democrats (SDP) support the creation of a technology council. But they want a much broader committee, with wider powers and members representing industry, politics and sci-

ence. It would be responsible for developing a coordinated strategy for science, and for deciding on the financial and administrative changes required for its implementation.

Krüger believes that two committees he is setting up will achieve most, if not all, of the aims of the proposed technology council. One is a 14-strong 'strategy circle', set up last summer and made up of representatives from industry and science. This advises the minister on how conditions for research and technology transfer might be improved, and brings together scientists and industrialists with common interests.

In parallel, Krüger is setting up a group of more sharply focused 'dialogue circles' (*Zieldialogkreise*). These will look at individual scientific areas and identify precisely where industry could benefit from basic research, trying to predict the products that could be developed so that more goal-directed science policies can be defined.

The SDP does not think Krüger is going far enough, and dismisses the strategy circle as a debating society with no influence over government policy. It accuses the government of allowing research funding in Germany to fall at a time when greater investment is needed to ensure competitiveness.

The SDP says that if it wins next October's elections, it will make good the shortfall, calculated at about DM1 billion. All three major parties agree on one thing: much more deregulation is required to make life easier for academic and industrial scientists. The ministry of research and technology is already examining existing and proposed laws that impinge on research, and Krüger says he will shortly create a special section within the ministry to concentrate its efforts. **Alison Abbott**

Lords warn industry and government to back science changes

London. A committee of Britain's House of Lords has warned the government that the benefits of last year's white paper (policy document) on the organization of science will materialize only if both industry and government do more to support a 'partnership' with the research community.

The report — from the Lords' Select Committee on Science and Technology — is being published one week after the Department of Trade and Industry (DTI) announced that it has abolished the post of chief scientific adviser. Under a reorganization taking effect on 1 March, scientific issues will be dealt with by the revamped industrial sponsorship division, and the department will concentrate its support for innovation on projects such as regional technology advice centres.

Lord Flowers, the chairman of the select committee (and a former chairman of the Science and Engineering Research Council), says that he has not been told of the reasons for the DTI's decision. But he admits that it appears to go "in the opposite direction" from what the committee is advocating.

"Until I have had a satisfactory explanation, I cannot help but regard the event as the removal of an office which forms a natural public focus for science and technology activities within a very important part of the government," says Flowers. "I cannot see the logic of it."

The committee's report welcomes the general thrust of the white paper in the need for the research community to focus more clearly on the priorities of British industry. It also applauds the appointment of a single individual responsible to the minister for running the six research councils, an innovation close to some of the committee's own proposals in the past for a single research council.

But it claims that, while the scientific community has been striving to build a partnership with industry for some time, there has been an inadequate response from the 'user' side. Flowers points to the relatively low level of investment in civilian research and development by many British companies compared to their overseas competitors, and to the need for their greater involvement in the process of setting the country's long-term technological priorities.

David Dickson

US panel wants global body to control civil plutonium use

Washington. Plutonium meant for use as nuclear fuel can readily be used for bomb-making, and therefore poses a large and growing proliferation threat, according to a major report published this week by the National Academy of Sciences in Washington (see also page 301).

As a result, the report — which was prepared for the president's National Security Adviser, and is expected to have a strong impact on the Clinton administration's proliferation policy — calls for all civil or reactor-grade plutonium to be brought under a new international regime to prevent its theft or misuse.

The panel, which was chaired by Wolfgang Panofsky, former director of the Stanford Linear Accelerator Centre, also recommends that the United States and Russia should negotiate a new agreement to jointly monitor and control the stockpiles of plutonium and other materials being created by the dismantling of nuclear weapons under arms control agreements.

It argues that the United States should offer immediate assistance to Russia and other former Soviet states to help with security at sites where weapons-grade fissile material is stored, in order to counter the growing risk of theft.

And — in a blow to the Department of Energy's troubled Advanced Liquid Metal Reactor at the Argonne National Laboratory (see *Nature* 365, 99; 1993) — it says that development of new types of nuclear reactor cannot be justified in terms of their potential for disposing of plutonium. Plutonium, it argues, is best burned without reprocessing

in existing nuclear power stations, or else stored in glass with other nuclear waste.

One of the clearest messages from the 280-page report, prepared by the academy's Committee on International Security and Arms Control (CISAC), concerns the looming danger of civil grade plutonium being used for bomb-making.

"Reactor grade plutonium can be used to make nuclear weapons," says the committee's chairman, John Holdren of the University of California, Berkeley. "Our report is quite explicit about, firstly, its usability in weapons, and, secondly, its extractability from spent nuclear fuel."

The report explains how problems about using reactor grade plutonium can be overcome by weapons designers, and warns that the extraction of such plutonium from spent nuclear fuel becomes easier as the fuel ages, multiplying proliferation risks.

It calls for "consideration of more proliferation-resistant nuclear fuel cycles", and although it does not make any direct recommendation on THORP, the huge nuclear fuel recycling facility due to start up in Britain later this year, it could make an impact on the debate in Britain.

"There is currently no economic case [for recycling]," says Holdren. "We make that clear, so we may have some impact on THORP because it will be harder for anyone to argue that it has an economic rationale."

In two month's time, the academy will report in more detail on reactor options for disposing of weapons plutonium, and a report on dealing with spent nuclear fuel will appear later in the year.

Colin Macilwain



Lord Flowers

Praise for Rutherford as its future is debated

London. Britain's largest government research facility, the Rutherford Appleton Laboratory, still provides 'state of the art' facilities despite a reduction of one-third in research council funding over the past five years. But a National Audit Office (NAO) survey found that in some cases demand for its services is three times what the laboratory can supply.

The government's watchdog body found the laboratory had successfully increased its external funding as direct grants from the Science and Engineering Research Council (SERC) had dropped, and had managed to maintain high standards. About 70 per cent of respondents to the NAO survey said that the laboratory had been "absolutely vital" or at least "very important" in achieving their research objectives.

Staff at the laboratory came across as

helpful, responsive and knowledgeable "beyond the call of duty". One respondent described them as "overworked and underpaid", and overall 95 per cent of respondents said they found facility staff good or excellent.

The main complaint was about lack of resources. Of the four key facilities, ISIS, the world's most powerful source of pulsed neutrons, came off worst, with 45 per cent of users saying competition for time and resources meant they had not received all they requested.

The NAO report comes at a time when the future role of the laboratory is under debate. From April, responsibility for the laboratory will transfer from the SERC to the new Engineering and Physical Sciences Research Council (see *Nature* 366, 100; 1993), but a more long-term shake-up is anticipated.

Fiona Gammie

Earthquake centre was victim of cash curbs

Washington. On 17 January, the very day of the Los Angeles earthquake, the National Science Foundation informed the Southern California Earthquake Center in the city that its annual grant is to be increased next year by \$1 million.

The NSF is hoping that the increase — whose timing is said to be entirely coincidental — will take the heat out of charges that the centre, which now serves as the focal point of scientific investigation into the earthquake, was badly underfunded.

The 'open-walls' centre, based at the University of Southern California in South Central Los Angeles, is widely seen as a good example of the type of interdisciplinary research centre favoured by government agencies as a way of applying university research to specific problems — in this case, the risk of secondary earthquakes in the Los Angeles area.

Like other similar institutes in both the United States and elsewhere, the earthquake centre was set up as part of an ambitious scheme to divert tens of millions of dollars into such specialist research centres. But this ambition was deflated by fiscal pressure and complaints from scientists who feared such centres would take money away from other, more worthwhile projects.

Although some West Coast seismologists say the centre originally asked the NSF for up to \$10 million a year, its first formal application was for a grant of \$5 million. The NSF initially responded with an offer of \$2.5 million; once the foundation had dealt with a crisis over the funding of its station in Antarctica, the earthquake centre actually ended up with \$1.4 million in 1991.

Over the past three years, that has grown to \$1.8 million, and the NSF will provide the centre with \$2.8 million next year, in addition to \$1.2 million from the US Geological Survey.

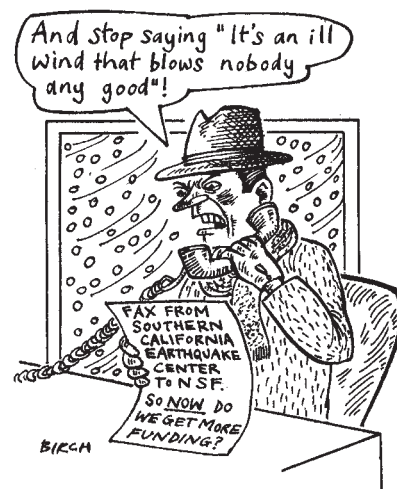
Tom Henyey, the centre's executive director, has more patience with the founda-

tion than some scientists in Los Angeles. He acknowledges that the NSF is torn between funding centres such as his, or providing grants for individual researchers. "They have to walk a fine line," he says.

The centre's goal is to construct a model of the area's seismology which is sufficiently accurate to allow for the probabilistic forecasting of earthquakes. Pursuing this goal involved bringing together geologists, seismologists, structural engineers and others for monthly meetings, for workshops on specific topics and for report writing.

Despite moderate funding — its budget is spread thinly among 50 principle investigators — the centre has attracted the participation of many earth scientists working locally. "We all come to the meetings," says Lucy Jones of the US Geological Survey at the California Institute of Technology.

"The centre is like a conscience," says Henyey. "A lot of earthquake preparedness is just getting the word out. It's not that we



are going to come up with some great discovery; but we are coming to a better understanding of the earthquake potential in the Los Angeles basin." **Colin Macilwain**

Russia holds on to science ministry

Moscow. Despite the resignation of one of its most powerful supporters, the former first deputy prime minister Yegor Gaidar, Russia's Ministry of Science appears to have survived an attack from the Russian Academy of Sciences, and to have maintained its independence in the new government of President Boris Yeltsin.

According to a decree issued by the Russian president, the ministry is one of 23 departments which will make up the new government. At present, it appears that the former Minister of Science, Boris Saltykov, will remain in his post, although his future is by no means certain.

The attack from the academy took the form of a resolution passed by its praesidium in December, which blamed Saltykov for

trying to destroy it. The resolution suggested that the ministry be replaced by a state committee — which could have allowed the academy to regain direct control of basic research activities which Saltykov has suggested should be funded independently.

But the academy's actions provoked a flow of letters to both President Yeltsin and his prime minister, Viktor Chernomyrdin, in support of the activities of the minister of science. In particular, one such letter was signed by the directors of nine of the country's largest scientific centres, describing the academy's attacks as "deeply erroneous".

The signatories of the letter included Spartak Belyaev, the director of the Institute of General and Nuclear Physics — the so-called Kurchatov Institute — of the Russian Scientific Centre; Igor Chuvila, director of the Institute of Theoretical and Experimental Physics; and Alexander Simonov, of the Karpov Physico-Chemical Institute.

The letter to Yeltsin endorsed Saltykov's attempts to move part of the funding of research projects on to a competitive basis, a move which had been sharply criticized by the praesidium of the academy.

Other letters criticizing the leadership of the academy were received from the heads of all three of its regional branches, in Siberia, the Urals and the Far East. In addition, members of the academy based in Novosibirsk have written a joint letter to its president, Yuri Osipov, demanding new elections for all members of the praesidium — a proposal which is expected to be raised at its next general meeting. **Vladimir Pokrovsky**

China promises to behave on patent protection

Beijing. China has promised to introduce full protection for intellectual property as part of its bid to rejoin the group of countries covered by the General Agreement on Trade and Tariffs. (GATT).

The promise was made last month by Gao Lu-Lin, director-general of China's Patent Office, when he briefed a meeting of patent officers from all part of the country on China's latest effort to rejoin GATT countries and to bring its patent laws in line with those that have been adopted by other countries.

In the past, China reluctance to apply Western patent laws has become an increasing source of friction in its trade

relations with the West, particularly the United States. In recent years, however, China has joined several international conventions on intellectual property.

Its officials maintain that the country has now fulfilled almost all of the intellectual property requirements necessary to be restored to its GATT membership.

"China must follow the GATT requirements if it wants to establish an economic presence in world markets," Gao Lu-Lin told last month's meeting. "Without international intellectual property protection, we will find it difficult to bring our technologies and products into world markets." **You Qin Li**

ANC plans to boost prospects for black science students

Cape Town. In preparation for South Africa's first democratic elections in April, the African National Congress (ANC) has published proposals for increasing the number of black students taking university courses in science, engineering and medicine.

The proposals are expected to be formally adopted at the ANC's policy conference in March, and are aimed at correcting the imbalance between the number of black and white students in higher education. One way of doing this, says the ANC, would be to establish a national higher education council with the power to regulate the distribution of enrolments across sectors, institutions and disciplines.

The existing funding formula would be replaced by one incorporating subsidies for academic development programmes for disadvantaged students — who do not at present receive any government support — and redress institutional inequalities by favouring universities that have received relatively low levels of funding in the past.

The chances of such changes being implemented are good, as most polls put the ANC's support at about 60 per cent of the population. Some suggest it may be as high as two-thirds, the level of votes in the National Assembly required to enact a new constitution.

Harold Wolpe, director of the University of the Western Cape's education policy unit, which was heavily involved in formulating the ANC's policy, says that the proposals also favour the gradual merger of universities and 'technikons' into a single sector, as has happened recently in the United Kingdom and Australia.

The merged institutions would come under the control of central government. In contrast, colleges offering certificates in teacher education, nursing, agriculture and technical subjects would, together with schooling, be the responsibility of the nine regional governments.

The proposals say that South Africa should not divide universities into graduate and research institutions, or introduce teaching-only universities. But they do support the idea of centres of excellence, with some rationalization of university departments at the regional level.

A single national department of education and training would coordinate all educational programmes, and flexible access between sectors would be made easier by a national qualifications structure.

With regard to financing higher education, the ANC proposes to replace the present system, in which all students pay fees, with one in which fees are charged on a means-tested basis.

Michael Cherry

Funding dispute could hold up biodiversity treaty

London. Britain's environment minister, John Gummer, this week released an 'action plan' on biodiversity — one of four documents setting out how the government plans to meet commitments made at the Earth Summit held in Rio de Janeiro in 1992.

Behind the scenes, however, the government is threatening to delay ratification of the UN Biodiversity Convention until a dispute is resolved about the procedures by which developing countries are to be funded to meet their commitments under both this and the Climate Change Convention.

At the centre of the dispute is the Global Environment Facility (GEF), a \$1.3-billion fund set up by the World Bank in 1990 to pay the marginal costs of conserving biodiversity, mitigating climate change, pre-



Home grown: most efforts to preserve biodiversity are small-scale.

serving the ozone layer and abating the pollution of international waters.

At the insistence of the member countries of the Organisation for Economic Co-operation and Development (OECD), the GEF emerged from the 1992 Earth Summit in Rio de Janeiro as the principal channel through which developing countries would be paid to meet obligations under the Biodiversity and Climate Change Conventions agreed at the meeting.

But the GEF — whose initial three-year pilot phase comes to an end in June — came in for some harsh criticisms at a meeting in Cartagena in Colombia last month, when an independent review team reported that it suffered from "inadequate" leadership and management, "complex, cumbersome and costly" decision-making processes and "diffused" accountability. The review claimed that developing countries were able to influence its operations "only on the margins".

British officials say they share these concerns, which have been voiced by government representatives and environmental groups in both developing and developed countries (including the United States).

But Britain and other OECD countries are insisting that the GEF must remain the

principal financial mechanism serving the UN conventions. They are also determined that plans for restructuring the facility in the light of the evaluation will still ensure that funds are spent in a way that they approve.

Officials from Britain's Overseas Development Administration (ODA) are therefore warning that Britain will not ratify the Biodiversity Convention until the GEF's future is secured to its satisfaction. "I am sure that an effective replenishment and restructuring of the GEF would give the necessary reassurance to UK ministers [to ratify the convention]," says David Turner, head of environment policy at the ODA.

But the resolution of the GEF's problems may still be some way off. In order to solve the problems identified by the independent evaluators, the countries represented at Cartagena agreed that the GEF should set up a secretariat independent of the World Bank, reporting to a new executive council.

Talks broke down, however, over the relative representation on the council of the developed donor countries and the recipient developing and East European countries. In particular, France threatened to cut 30 per cent from its contribution to the \$2-billion replenishment target for the GEF's second phase if OECD representation fell below half of the 30 seats.

Following further discussion, the OECD countries have confirmed their commitment to the \$2-billion target, and expressed their desire to resume negotiations with the developing nations. As a result, talks on the future of the GEF are due to resume in February. One possible compromise is a 31-seat executive council, in which the recipient countries would have 16 seats. But the outcome is still uncertain.

Critics of Britain's position argue that it is trying to use the GEF to limit its support for international efforts to preserve biodiversity. "The financial obligations and opportunities created by the Biodiversity Convention go far beyond those offered by the GEF," says Tony Juniper of Friends of the Earth.

Alistair Graham of the Biodiversity Coalition, based in Australia, claims that, even with its new status, the GEF will still be almost entirely run by World Bank staff used to overseeing large development projects, and lacks sufficient skills and experience to support small-scale conservation projects run by local communities.

Graham and others are urging the creation of a new fund directly controlled by the signatories of the Biodiversity Convention — precisely the type of move that Britain, and several of its OECD partners, are determined to avoid.

Oliver Tickell

Evaluation of Spanish research

SIR — The report by Oscar G. Segurado (*Nature* 366, 9; 1993) on the Spanish Biological Research Centre (CIB) must be seen in relation to the new policies on evaluation and reorganization being implemented in the Spanish Council for Scientific Research (CSIC) as a whole.

There have been important changes in Spanish science and technology since 1983, notably new laws regulating universities and public research organizations. At the same time, resources for research have increased considerably; between 1983 and 1990, the percentage of gross national product devoted to research and development rose from 0.6 to 0.92, the number of researchers increased from 1.0 to 2.5 per 1,000 of active population and there are new procedures to evaluate the scientific activity of researchers and research groups.

The CSIC, created in 1939, is the largest public research organization in Spain and covers almost all fields in the natural and social sciences as well as the humanities. CSIC researchers number 6 per cent of the Spanish total, but their output is 20.6 per cent of the total.

CSIC has sought to adapt to all these changes by a new set of rules (adopted a year ago) aimed at a more efficient and flexible organizational structure, improving scientific performance and facilitating cooperation with universities and other research institutions. Research institutes and centres are now considered the most important parts of CSIC's structure. Their directors now have the power to organize and supervise their activities. They have more power than before, but the researchers and staff personnel directly participate in the election of the directors within the institutes and centres. There is also a procedure allowing institute members to monitor management decisions. These changes in the directors' role were necessary to achieve more efficient management.

Before the approval of the new rules, most of the scientific personnel consulted accepted these changes. In the past three years, evaluations have been carried out affecting 28 institutes, 500 researchers, 900 staff members and 400 graduate students. As a result, five institutes have been closed down, six merged, four reorganized and six new ones created. Decisions have not yet been taken in the remaining cases.

Thus, the CSIC, like any other research institution, must solve problems arising from reallocation of scientific personnel within or between institutes. A detailed study is made in order to adopt the most convenient solution, and to avoid creating additional problems that might interfere with the normal development of the re-

search work. The first solution proposed is not always adopted; the advisory and executive bodies examine alternative solutions that may be more appropriate. In the case of the CIB, the decision fits in better with these criteria and was not taken in response to pressure from any scientific union.

The CSIC stands firm in its intention to implement new evaluation and reorganization policies for its institutes and centres. What occurred in the CIB must not in any way be understood as a retreat or a threat to its position. It is simply one more case where these new policies have been implemented to improve the efficiency of our organization.

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SIR — Segurado points to important elements of concern about the future of research institutions in Spain. Although it is obvious that the new CSIC rules increase the authority of the president and of the institute directors, the president has always been able (and continues to be able) to boost particular fields of research in the most effective way, by deciding on the distribution of new positions (including those for internal promotion) among the different disciplines and by directly appointing the members of the selection committees.

Moreover, external evaluations are not new in the CSIC. The Cajal Institute, for example, to which Segurado refers, avoided dissolution in 1982 after two officially appointed external referees recommended support. The institute has had an external advisory committee ever since, but the evaluation in January 1992 was an internal one. It is ironic that such a delicate decision on personnel policy was taken after only an internal evaluation and so late in the day.

The CIB's external evaluation committee has now, according to Segurado, pointed out significant deficiencies that, interestingly, were also detected in the Cajal Institute by its external advisory committee several years ago. Leaving aside the urgent need for appointing new, well-trained scientific personnel, the committee also calls for breaking the isolation of the research groups and for defining common strategies.

Letters submitted for Correspondence should be typed, doubled-spaced, on one side of the paper only.

These objectives will hardly be attainable if the new CSIC rules are applied in the coercive manner described by Segurado. They may be fulfilled, however, if the CSIC encourages the application of the positive aspects of the new rules which, among others, include more efficient mechanisms to increase mobility between the CSIC and the universities.

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Setting the record straight

SIR — Luis Angel Fernández's News article (*Nature* 365, 684; 1993) may give a misleading impression.

He says that, in 1990, problems encountered in the course of an experiment involving genetically engineered plants carried out by the Catalan government's Institute of Agriculture meant that the crop being experimented on had to be burnt.

The Institut de Recerca i Tecnologia Agroalimentàries or IRTA (Institute for Food and Agricultural Research and Technology) is a public-sector company belonging to the Catalan government whose main activity is in the field of scientific research and technology transfer relating to agriculture and food. We therefore feel that "the Catalan government's Institute of Agriculture" will be understood as being IRTA.

The experiments referred to in the article were carried out in 1988 in the context of a research contract with the Belgian company Plant Genetic Systems under the direction of Dr Pere Arús, head of our department of plant genetics.

This contract was concluded on the basis of a research agreement approved by both parties which laid down and programmed not only the manipulation of the vegetable material, but also its subsequent destruction by boiling or burning. All the statutory requirements laid down by the relevant authorities (Ministry of Agriculture of Spain and Department of Agriculture of Catalonia) were observed.

At no time did anything occur that was untoward or that might have caused any risk or harm. The contract was fulfilled, the vegetable matter was destroyed as stipulated and the findings were in line with expectations.

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Still more about AIDS

SIR — G. Garnett and R. Anderson (*Nature* 366, 716; 1993) state that Duesberg's claim that drug use rather than HIV is the cause of AIDS has been firmly rejected, but we do not agree. They rely in part on the Vancouver study (*Lancet* 13 March 1993) but Duesberg has pointed out that that study does not provide controls (verified drug-free AIDS cases), does not quantify drug use and ignores Zidovudine use.

Garnett and Anderson also refer to one of us (J. S.) as a "television presenter" and use the phrase "non-expert opinion". J. S. has specialized in AIDS issues for seven years, and has made seven television documentaries analysing scientific knowledge about AIDS.

Our work in preparing for the British network television documentary referred to involved consulting expert opinion from all the countries we visited as well as that of our special consultant Dr Harvey Bialy. The remarks by Garnett and Anderson about Bialy are totally unjustified. Bialy spent six years working in West Africa on the epidemiology of plasmid-mediated antibiotic resistance among enteric pathogens, and in recent years has travelled extensively in Africa for WHO, UNESCO and UNIDO. He is an acknowledged expert on the implementation of biotechnology in Africa and has a wide knowledge of biotechnology-produced diagnostic tests.

Joan Shenton

Volker Gildemeister

Meditel,

Bedford Chambers,

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■ This letter was submitted to the *Sunday Times* on 16 December 1993 but was not published.

SIR — The truth about AIDS is to be found in the overall consensus, gained over years of research. This is the approach we take in studying any disease — not by homing in on one or two unusual or anecdotal reports, which has been the hallmark of the *Sunday Times* coverage of AIDS. Let us look at assertions made in the article "AIDS — why we won't be silenced" (12 December 1993).

■ US infection rates at 1 million are stable — "a new or mutant virus does not behave in this way". This is an odd statement. Infection rates rose rapidly in the mid-1980s in every group studied, as in the United Kingdom. Levels are stable now because the number dying of AIDS each year in the United States (more in 1993 than died in the entire Vietnam War) is now the same as the number of new infections. The rest can be explained by saturation effects in high-risk groups and

behavioural change. African and Asian infection rates have risen rapidly too.

■ It is true that almost all these deaths will be among homosexual men or drug abusers, many of whom are black. If, as some suggest, these deaths will have little impact, then that is a terrible reflection on an intolerant society rather than saying anything about the dangers of HIV and AIDS.

■ "In every patient studied so far there is never more than one in 1,000 cells infected with HIV". Out of date. There are high levels of HIV-infected cells in lymph nodes, even in early infection.

■ "Many healthy people have co-existed harmlessly with HIV for many years." Of course they have. This is a slow virus, but to survive with HIV longer than 15 years without signs of illness or immune damage is very rare.

■ "Even Luc Montagnier, who discovered HIV, has argued that microbes called mycoplasma, which he now knows to have been present in his original 'AIDS virus' culture, are the chief cause of the death of the immune cells in AIDS patients." Again, nonsense. No one gets AIDS from mycoplasma alone. Mycoplasma infection is very common in the general population.

■ "The evidence HIV causes AIDS is still epidemiological." Of course. How else can one find a link? For the link between smoking and cancer, we accept such evidence as valid, and rightly so — why the double-think when it comes to AIDS?

■ People with HIV die of AIDS, those with no HIV do not. Those with haemophilia, or those who inject drugs with shared needles, or homosexual men can be separated into two groups on the basis of an HIV test. Identical in all other respects, one group becomes ill and dies with AIDS, the other remains healthy.

■ It is true that out of thousands of cases of severe immune deficiency studied, very occasionally someone has been found with an illness that looks like AIDS in the absence of HIV. So what? Doubting the HIV link on that basis is as stupid as saying that, because lung cancer can happen in non-smokers, smoking does not cause lung cancer.

■ We are told that haemophiliacs with HIV who have converted to using purified blood products "have recovered fully . . . and there now seems no reason why they should not have a normal lifespan". There is no evidence to support such a wild statement.

■ Finally, we are presented with a paper in *Biotechnology* claiming that a high proportion of those testing HIV positive in Africa are "false positives because of the inadequacies of the test". It is a pity that no one at the *Sunday Times* bothered

to check the paper's references. If they had, they would have found that the evidence of false positives is dated almost entirely from the mid-1980s, using the very first primitive tests for HIV. These did have high false positive rates, unlike the tests used for the past few years.

Recently, a *Sunday Times* banner headline read "African AIDS Plague a Myth". This sensational story was based on a conversation with a pair of charity workers in Tanzania, neither of whom is a doctor or scientist. Their anecdotal accounts were presented as fact, while local experts were ignored. Their credibility is very low in Tanzania. The misquotation from Dr Timothy Stamps of the Department of Health in Zimbabwe, apparently supporting the 'AIDS myth' theory, was wildly misleading and poor journalism, as he is well known for his view that AIDS is a serious threat in Africa and elsewhere.

How can you write off the whole African AIDS epidemic as a "myth" when six out of ten women with AIDS in London are from African nations, infected abroad — the visible edge of a huge epidemic? Most specialists are convinced that 90 per cent of new infections are heterosexually transmitted worldwide, more than half among women.

I am convinced that each one of these poorly researched stories has cost lives. You have created confusion and encouraged carelessness. I am all for balanced scientific debate but this, quite simply, is poor, but dangerous, journalism.

Patrick Dixon

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Poverty not a cause

SIR — In commenting on India's latest earthquake (*Nature* 365, 476; 1993) you emphasized that it was not the earthquake *per se* but poverty that killed people. However, instead of revealing the underlying socio-economic and political dynamics of poverty along the lines of seismic mechanisms of earthquakes, the discussion was devoted to the ramifications of poverty and their ill-effects. Why only earthquakes? Scientists are equally fond of blaming poverty for a similar role in flood, famine and epidemics such as AIDS. But where does poverty come from, and who perpetuates it? Unless such questions become part of scientific discourse, poverty will continue to be construed as a 'cause' rather than a 'product', and its resolution will remain elusive.

L. R. Murmu

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Towards ageism

SIR — There is more to the ethical debate about postmenopausal births than the sanctimony of the British press (*Nature* 367, 2; 1994) or than you allow. Of course, many of the populist arguments fail by extension. For example, if a 59-year-old woman should not have a child because of the risk of her early death, then what of a mortally ill 29-year-old who wishes nonetheless to conceive naturally? Stressing "what is fair to the child" has been a common theme, but both these circumstances are equally unfair to the child and are ethically equivalent.

It is more arguable whether society has a duty to enable conception by women who are not fertile, or not willing to conceive in the usual way. If society is asked to provide the means to conception, then this 'right' can no longer be consi-

dered in isolation, which provokes the real ethical question raised by *in vitro* fertilization — equity.

The world is quite plainly an inequitable place. It is unrealistic to demand Utopia, but most high-technology medicine increases that inequity. Attempts to prolong by medical means the natural fertility of women while so many in the world have no hope of seeing their naturally born children grow to adulthood is another way of increasing the inequity between the haves and the have-nots. The aim of medicine should be to enable people to live their natural lives as comfortably as possible, not to enable them to have whatever they want, whenever and for as long as they want it.

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Legalizing drugs

SIR — Most of us involved in research on marijuana agree (with a few vocal exceptions) that it is a relatively safe drug. The comparison in your leading article "Decriminalizing drugs?" (*Nature* 366, 598; 1993) with alcohol seems relevant, and there is some scientific support for this. Perhaps we should give serious consideration to the comments of the Surgeon-General of the United States that the legalization of drugs might lead to a reduction in crime, and not allow emotional reaction and vested interests to carry the day.

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Appeal to Mitterrand in blood scandal

■ This is the text of the letter sent last week by 97 researchers, physicians and public health workers to the French President, François Mitterrand, asking him to pardon the four physicians convicted last year for their part in the events surrounding the contamination of blood supplies in the mid-1980s with human immunodeficiency virus (HIV):

M. PRESIDENT — A French court has sentenced four doctors for the decisions and the measures that were collectively taken by various authorities in 1985 to stop contamination of haemophiliacs with the AIDS virus. Two of these doctors are in prison. One of them is the director of a major blood transfusion centre, the other a scientist internationally recognized for his contribution to AIDS research.

We, doctors, scientists and public health workers from around the world, some of whom are personally affected by this drama, think that it is our duty to express our feeling of injustice and our concern about the possible consequences of this verdict on public health and biomedical research.

The verdict on these doctors was delivered after an unprecedented 'trial by the media', where sensationalism dominated to the detriment of accuracy, and passionate debate took the place of objective facts. Thus, in the absence of extended scientific expertise, punishment was opposed to the uncertainty of scientific knowledge at the time of the facts. We believe that such a verdict is unfair, and an inappropriate way of dealing with suffering born of scientific uncertainty. We also believe that it is deeply unjust to punish a few selected individuals for the lacunae and dysfunctions of our health system as a whole.

Furthermore, this verdict is worrying in that, under the influence of similar outside pressures, it could happen again in other cases involving decisions made on matters

at the frontiers of scientific knowledge. Such sentences work against medical progress, because the fear of legal reprisals could then divert doctors and scientists from doing their duty and from taking the responsibilities that are theirs. As a result, medical research and patients would be deprived of the valuable contribution of devoted, highly skilled professionals.

The extent of infection of haemophiliacs with the AIDS virus in France is unfortunately analogous to that in other countries where the same therapeutic approaches were used. As in other countries, most contamination cases occurred at the beginning of the 1980s, when science, medicine and technology were not yet able to prevent them. Throughout the world, thousands of doctors and scientists were involved in the preparation and administration of anti-haemophilic factors, and in most countries undeniable delays occurred in the decision-making processes concerning this particular public health problem at various administrative, technological and clinical levels. Is it reasonable in such circumstances to attribute blame to a few individuals in the face of an almost universal health hazard, which involved so many men and women whose very aims in life are to help and to heal?

Through numerous examples, the history of medicine has taught us that months, and often years, must pass before a scientific discovery is validated, recognized and integrated into the general consciousness. Only then can such discoveries be brought to bear upon decisions concerning public health. Humility, one of the prerequisites of progress, obliges us to recognize that, despite its fantastic evolution, the power of medical science is still less great and less immediate than popular hope and imagination would care to acknowledge. Our public health structures still have a long and arduous path of development and maturation ahead.

While affirming our solidarity and compas-

sion for the haemophiliacs and their families stricken by AIDS, it is our duty to guard against any unjustified sentiment of mistrust against doctors and medical science, which would be counterproductive to public health. Thus, it is for the sake of public interest, M. President, that we wish to share our serious concern with you.

We hope that it will be possible for you to use your authority and power justly to appease past and present suffering and to prevent any wrongs that science and indifference might bring about in the future. A first step in this direction would be a presidential pardon.

MAURICE ALGAZI, ROSEMARY ANCELLE-PARK, BIRGITTA ASJO, FRANCIS BARIN, MICHEL BARME, FRANCOISE BARRE-SINOUSSE, HENRI BAYLON, JOSEPH BENICHO, GABRIEL BENICHO, ANNIE BEZEAUD, ANTOINE BLANCHER, WILLIAM A. BLATTNER, PHILIPPE BLOT, GEORGES BOURROUILLOU, DAVID BOUTTIER, BERNARD BOVEN, JEAN-BAPTISTE BRUNET, YVES CADROY, JACQUES CAEN, CHRISTIANE CAHAN, PATRICK CALVAS, CLAUDINE CARANOBE, ROBIN W. CARRELL, MICHEL CHANZY, GERARD CHAQUAT, FRANCESCA CHIODI, PIERRE COLOMBIES, ANTONIO COUTINHO, JEAN DAUSSET, ISABELLE DE VINCENTI, PATRICE DEBRE, FRANCOISE DENIS, JAN DESMYTER, VINCENT DEUBEL, AGNES DEVERGIE, ANDRE DODIN, JEAN-FRANCOIS DORE, JEAN-CLAUDE DREYFUS, COLETTE DREYFUS-BRISAC, JEAN DUCOS, HERVE DURAND, EVA MARIA FENYÖ, HERVE FLEURY, JEAN-JACQUES FOURNEL, JACQUES FROTTIER, JEAN GAILLARD, PIERRE GALANAUD, PIERRE GARCON, LOUIS GAZZOLLO, ALAIN JEAN GEORGES, MARIE-CLAUDE GEORGES-COURBOT, MARC GIRARD, ELIANE GLUCKMAN, JEAN CLAUDE GLUCKMAN, MICHEL GOLDBERG, PATRICK GOUBAU, MARIE-CLAUDE GUILLIN, ANDRE HERRAULT, MYRA JENNINGS, IRENE JUHAN-VAGUE, PHYLLIS J. KANKI, MICHEL KAZATCHKINE, ANDRE KIRN, DAVID KLATZMANN, JEAN-PIERRE KOHEN, HENRI KREIS, JACQUES KRUIH, LEON LE MINOR, GUY LESOURD, ROSETTE LIDEREAU, PIERRE LORTHOLARY, WILLIAM LOWENSTEIN, JEAN LOYQUE, HERBERT MARCOVICH, MICHEL MARTY, CHRISTIAN MATHIOT, PHILIPPE MONOD-BROCA, GERARD ORTH, LUIZ PEREIRA DA SILVA, GEORGES PERES, JACQUES PILLOT, CHRISTIAN POLICARD, JACQUES REYNES, WILLY ROZENBAUM, GABRIELLA SCARLATTI, GERARD SCHAISON, JORG SCHUPBACH, PIERRE SIE, WINSTON SMILOVICI, JEAN-CHARLES SOURNIA, DANIEL TARANTOLA, ARMAND TAVITIAN, ALAIN TEDGUI, PIERRE TESTAS, PIERRE TIOILLAIS, AGNES ULLMAN, ELIE WOLLMAN, ALICE WOLLMAN.

Health decline in Eastern Europe

Richard Feachem

Large relative and absolute declines in health status in Central and Eastern Europe since the mid-1960s place the countries of this region at a considerable disadvantage as they strive to compete and participate in the new Europe.

THE quality of the human capital that the post-Communist societies of central and eastern Europe have inherited can be measured by health status, educational attainment, and by other characteristics. Despite the rhetoric of the former Communist regimes, the emerging reality is that the health status of Central and Eastern Europe is extremely poor and has fallen far behind that in Western Europe in the past 30 years.

In describing and analysing this situation, three approaches are helpful: to examine current health status in relation to wealth (are the people of Central and Eastern Europe more or less healthy than their wealth predicts?); to examine the historical trends throughout Europe over the past 40 years; and to consider how the gap in health status between East and West might be closed. (The countries of Central and Eastern Europe, referred to here as the former socialist economies of Europe, are listed in the table. They

exclude the southern republics of the former Soviet Union.)

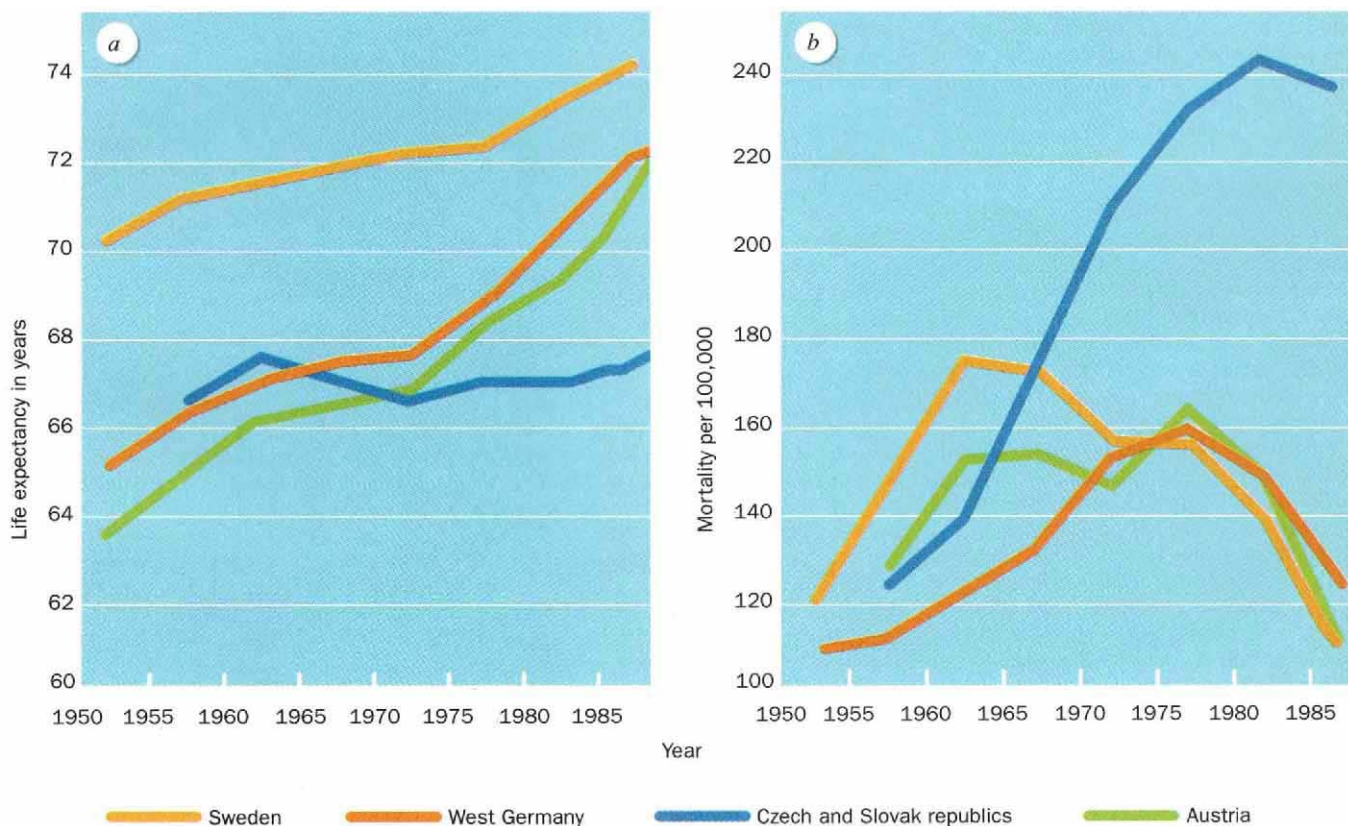
Health and wealth

The relationship between GDP per capita and life expectancy at birth is strong, especially at lower levels of GDP per capita. The life expectancy at birth in the former socialist economies of Europe varies from 69 years in Moldova to 73 years in Belarus and Bulgaria. Countries with similar GDP per capita — such as Chile, Malaysia and Venezuela — have life expectancies at birth of 71–73 years (ref. 1).

Risk of death between birth and the fifth birthday (child mortality risk) is also similar in the former socialist economies of Europe (average 2.2%) to that in countries with equal wealth. But for adult male mortality risk (the risk of death between 15 and 59 years of age) the former socialist economies are much less healthy than their wealth would predict.

Adult male mortality risk in the former socialist economies ranges from 20 (former Yugoslavia) to 31% (Hungary), with a regional average of 28%. By contrast, countries of similar wealth have adult male mortality risks of 18–21%. China, a much poorer country, has a risk of 20% and the Middle East and north Africa, also poorer regions, have a regional figure of 23%.

It is well known that the relationship between life expectancy and GDP per capita is weak at higher levels of GDP per capita¹. It is thought that, among the industrialized countries, income differentials are better predictors of health status, with more equitably distributed wealth being associated with greater longevity². By this criterion, the former socialist economies of Europe should have a considerably better health status than countries of similar wealth and, in particular, they should have notably better health than countries in Latin Amer-



a, Male life expectancy at birth 1950–88. b, Mortality from ischaemic heart disease in males 45–54 years old, 1950–88. (Both graphs are redrawn from ref. 3.)

COMPARISON OF HEALTH STATUS IN THE FORMERLY SOCIALIST ECONOMIES AND THE ESTABLISHED MARKET ECONOMIES, 1990

Health measure	FSE	Established market economies
Life expectancy at birth (years)	72	76
Life expectancy at 15 years (years)	59	62
Risk of death between 0 and 4 (%)	2	1
Risk of death between 15 and 59 (%)		
Males	28	15
Females	11	7
Doctors per 1,000 population	4.7	2.5
Hospital beds per 1,000 population	11	8

The former socialist economies (FSE) consist of Albania, Belarus, Bulgaria, Czechoslovakia, Hungary, Lithuania, Moldova, Poland, Romania, Russian Federation, Ukraine and Yugoslavia.

Established market economies are Australia, Austria, Belgium, Canada, Denmark, Finland, France, Germany, Greece, Italy, Ireland, Japan, Netherlands, New Zealand, Norway, Portugal, Spain, Sweden, Switzerland, United Kingdom and United States.

(Data from ref. 1.)

ica. For example, in 1989, the percentage of household income received by the poorest 20% of households was 11% in Hungary and 5% in Venezuela, and the percentage received by the richest 20% of households was 34% in Hungary and 50% in Venezuela¹.

Trends since 1945

After recovery from the Second World War, the health status of the former socialist economies overlapped with that of Western Europe. For example, life expectancy at birth in Czechoslovakia was higher than in Austria throughout the 1950s and 1960s (*a* in the figure). Furthermore, in the decade from the early 1950s the improvements in health status in the former socialist economies of Europe outpaced the performance of most OECD countries except Japan. Infant mortality rates fell by nearly one-half in the Communist countries, and life expectancy at birth increased by around 5 years. By contrast, life expectancy over this period increased by 2.5 years and 1 year in West Germany and the United States, respectively. By the mid-1960s, only 1–2 years separated average life expectancy in the former socialist economies of Europe compared with the advanced capitalist countries, and the gap was closing⁴.

From the mid-1960s, the relative trends in Europe changed dramatically. Health status in the former socialist countries stagnated or deteriorated, whereas in the OECD countries it improved steadily. Thus, between the mid-1960s and the late-1980s, life expectancy at age 1 fell for males in all the former socialist countries (greatest fall: Hungary, 3.5 years) and rose by less than 1.5 years for females over the same period. The decline in life expectancy in the Czech and Slovak republics relative to Austria, former West Germany and Sweden is shown in the figure (*a*). Age-standardized male mortality rates in Bulgaria, Czechoslovakia, Hungary and Poland rose by 2–13%, while in Netherlands, Sweden, Switzerland and the

United Kingdom they fell by 12–27% (ref. 4).

The widening gap in life expectancy and mortality between East and West was particularly striking in middle-aged adults. For example, in European Communist countries, death rates for males aged 45–49 years increased by between 7% (East Germany) and 131% (Hungary) between 1965 and 1989, whereas at the same time they were decreasing in the OECD countries⁴. A substantial proportion of this divergence in health status between the former socialist economies and the OECD is attributable to an epidemic of cardiovascular disease, and particularly of ischaemic heart disease, in middle-aged males. By the mid-1980s, mortality rates from ischaemic heart disease in men aged 45–54 years were twice as high in Czechoslovakia than in Austria, whereas in the 1950s they had been the same (*b* in the figure).

The gap with the West

It is no consolation to people in the former socialist countries of Europe to know that their health status is only a little worse than that of Malaysia or Venezuela, since it is not with Malaysia or Venezuela that they must compete. The need in these countries is for human capital stock that allows them to compete effectively with the countries of the European Union. At present the gap in health status between the former socialist economies and Western Europe is wide, and is especially wide in the male cohorts of working age (see table). Indeed, risk of death between 15 and 59 years for men is higher in Hungary than in Zimbabwe and higher in the Czech and Slovak republics than in Vietnam⁵.

The gap in health status portrayed in the table is due only to mortality differences. These mortality excesses place a burden on the economies of these countries because of lost investment in human capital (those who die in middle age will have been in receipt of publicly funded educa-

tion and other services), and because of medical investments made before their deaths. An even greater burden is likely to be imposed by the huge morbidity differential that underlies the mortality differential. Lost productivity due to high sickness rates in the labour force, as well as lost investment due to the high costs of caring for the chronically ill, put the former socialist economies of Europe at a striking disadvantage when competing with their healthier neighbours.

Causes of the gap

The data on wealth and health might suggest that the gap in health status between the former socialist economies and Western Europe is entirely due to differences of wealth and that, as the economies of the former socialist countries gradually strengthen, the gap will close. Such an assumption would confuse association with causation.

The cause of the gap has yet to be fully explained, but may lie substantially in lifestyle risk factors of which tobacco abuse, diet, alcohol and obesity are likely to be prominent. Comparative data show that, with regard to each of these factors, the former socialist countries are seriously disadvantaged compared to the established market economies⁶: tobacco alone may account for half of the gap⁷. Environmental pollution has also been blamed. Recent analysis suggests that air pollution may account for up to 9% of the gap in mortality between the Czech Republic and Western Europe, and a lesser proportion in other former socialist countries¹. Psychosocial factors may also be involved⁸. Lack of doctors or of hospital capacity is clearly not the problem (see table).

Repairing the damage associated with the past decades of Communist rule, and closing the gap in health status, must be a central objective for human-resources policy throughout the region. Improvements will be neither easy nor quick. If rates of overall mortality decline are similar to Chile's over the past two decades (roughly 2% per year), it will take East Germany 12 years to catch up with West Germany, and Hungary will need 23 years to rejoin Austria. □

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Molecular biology for general readers

Popular books on DNA are replicating quickly, but there is a case for taking seriously a distinctive (and Russian) view of what makes this molecule more interesting than others.

PEOPLE should not review books by their close friends, and especially not books by particularly close friends. There are two hazards. First, one may offend the friend, perhaps by offering gratuitous criticism of some element of the book of which he or she is particularly proud, but otherwise (and almost inevitably) by overlooking some such. The second and more serious is that readers in the know will write off the review as a "puff" and promptly think the worse of its author and even of the journal in which the review appears. All that is intended to disarm both categories of potential critics of what follows.

The book concerned is also referred to briefly elsewhere in this issue (see page 330), where David Lilley says he "found it quite hard to put down", but volunteers little else about it except that he "couldn't seriously recommend it to university students". The author is Maxime Frank-Kamenetskii, until last October a member of the faculty of the Russian Academy's Institute of Molecular Genetics in Moscow, but now translated to Boston University. The book is a slim volume called *Unraveling DNA* and published by VCH Publishers.

A few words of explanation may be helpful. Frank-Kamenetskii is a physicist by origin and thus representative of the Russian school of molecular biologists whose first knowledge of biology may have been culled from Schrödinger's *What is Life?*. It is not in the least surprising that so much of Russian molecular biology has origins such as these. Indeed, the Institute of Molecular Genetics is the outgrowth of a unit set up within the Kurchatov Institute (responsible for reactor design, among other things) by an enlightened director alarmed at Lysenko's influence.

An earlier version of the book appeared in Moscow nearly a decade ago under the title *The Most Important Molecule*, and was apparently a great success. There are heroic tales of how the English translation was produced in several consecutive all-night sessions in the kitchen of the translator, Lev Liapin, whose wife, Lydia Shepilova, used to manage the production of *Scientific American* in Moscow. The intended readership is the man or woman in the street.

As such, the book is distinctive because it tells the remarkable (and brief) history of molecular biology as the succession of fits and starts that we all recognize in retrospect. It is indeed the case that there was a minor exodus from molecular biology in the 1960s when, with the mechanisms of replication

(of DNA) and translation (of DNA into RNA) sketched out, and with the essence of the genetic code established, some thought the time had come to pay attention to other problems, the nervous system perhaps.

Then, within a year or so, there was reverse transcriptase (for turning RNA into DNA), which Frank-Kamenetskii says was less remarkable for its ideological significance than as a tool for the genetic engineers who quickly trooped along, stitching stretches of complementary (to RNA) DNA, now cDNA, into bacterial plasmids to manufacture the corresponding proteins. Since then, of course, there has hardly been a pause.

With all that said, this is a physicist's tale not only because it documents the antecedents of molecular biology in physics (Astbury, Bragg, Delbruck, Schrödinger and the like) but also because it abounds with illustrations of the kind physics teachers are forever using with their classes. The helical coil of DNA in a single human cell would be two metres long if fully stretched, right? But that is a factor of 10^6 greater than the dimensions of a cell nucleus, in which all the DNA is packed.

So can the compactness of the chromosomes be explained by random packing of the DNA, as in a ball of tangled wool? Sadly, a random walk calculation (made accessible by a tale of a man lost in a wood) shows that only a factor of 1,000 can be recovered that way. So there must be devices such as supercoiling, not to mention nucleosomes (made of histone protein) on which these coiled structures are wound.

Frank-Kamenetskii's other compelling rhetorical technique is to pretend for the benefit of readers that readers are intelligent beings. Take, for example, the difference between the genetic code that holds sway in the nucleus of a cell and that common to mitochondria. If mitochondria are indeed the relics of primitive prokaryotes that once formed symbiotic relationships with others to form now recognizable organisms, would one not expect the mitochondrial version of the genetic code to be the primordial version?

One would indeed, but one would be mistaken. Simple logic dictates that. With the transfer of some mitochondrial genes to the nucleus after the original symbiosis, it is just as likely that the mitochondrial code may have freed afterwards to evolve by the modest amount required to account for the observed difference. The interest of this technique is that it draws even general readers into current controversies, leaving them not with a flat statement that some

question is still decided, but with a vivid curiosity about the outcome.

Frank-Kamenetskii's own chief interest is in the structure of DNA. Sometimes, in conversation, he gives the impression that if only people were to think more carefully about the implications of what is known of the structure of DNA for the behaviour of the molecule in real life, much that is at present obscure would be understood. That interest no doubt explains the account of the role of supercoiling in the functioning of circular DNA molecules. Mathematicians (particularly topologists) are given full credit, as is James Wang of Harvard, the pioneer of the idea that topological invariants have a place in molecular biology.

The point of all this, in a book for general readers, is straightforward. Inevitably it is tempting, in describing progress in this field, to create the impression that everything has been so successful that there are hardly any problems left. But the truth may often be quite the opposite; the more successful and the more quickly moving some series of studies may have been, the more loose ends will probably have been left untied. But supercoiling is at least a way of accounting for the prevalence of the weird elements of nucleic acid structure such as the hairpins and the cruciform structures known to be prevalent in RNA molecules and likely, Frank-Kamenetskii guesses, in DNA structures as well.

As popular books on DNA do, this one ends (perhaps too suddenly) with an account of how molecular biology may be exploited for useful purposes in the years ahead — understanding cancer and perhaps curing (by gene therapy) some forms of it, and so on. But it is a proper question to ask whether, at the end of an essay in which readers have been half-persuaded that they too could be armchair molecular biologists by taking sufficient thought, that is the best place to leave them.

Much earlier, Frank-Kamenetskii has a chapter called "Where do genes come from?" which rehearses some of the questions commonly raised about the origin of life, but which is mostly an opportunity to explain that many genes have apparently meaningless introns scattered through their length, and that the genes of the immune system's ingredients are capable of rearrangement. But in reality, the most arresting question for the man or woman in the street is that of how life itself began. What molecular biology has done is to make even that question accessible.

John Maddox

A glimpse of heavenly features

Joseph Silk

THE detection of fluctuations in the cosmic microwave background by the COBE satellite, announced in 1992 (ref. 1), was a cosmological milestone. The discovery linked the very early Universe to the present-day large-scale structure in the galaxy distribution. Yet the sky maps that accompanied front-page banner headlines around the world were mostly of instrumental noise, rather than a display of

fluctuation spectrum and provides insight into its origin. The fluctuations were laid down close to the beginning of time, within the first 10^{-35} seconds of the Universe.

The growth of large-scale structure occurs by the operation of gravitational instability on infinitesimally small primordial density fluctuations in the expanding Universe. The application of this theory of

boost to fluctuation growth, and the expected amplitude of seed fluctuations was reduced until it could drop no further. Our cherished beliefs in the formation of structure out of the expanding Universe, combined with deep surveys of galaxies that monitored the present-day levels of density fluctuations, set a predicted level of temperature fluctuations (δT) that was now unambiguous. $\delta T/T$ could not be below about 5 parts per million.

Great jubilation greeted the COBE detection of fluctuations at roughly twice this level. There had already been extensive indications of 'extra' power on large scales out to about 100 megaparsecs, from deep galaxy surveys and peculiar-velocity studies. The microwave background fluctuations sampled scales in excess of 500 Mpc. Although the COBE fluctuation spectrum is consistent with the inflationary cosmology prediction, $\delta T/T \propto \theta^{(1-n)/2}$ on angular scale θ , with $n \approx 1$, the amplitude of the detected fluctuations meant that canonical cold dark matter was no longer a tenable model. There was now additional confirmation that some revision of the simplest theories of the fluctuation distribution and its connection to luminous matter was needed.

One proposed variation on this theme includes mixed dark matter, a combination of cold dark matter with a 30 per cent hot component in the form of neutrinos with rest mass of about 8 electronvolts³. Another is a vacuum-dominated Universe, in which the Universe is at approximately two-tenths of the critical density, but the cosmological constant, reincarnated as a vacuum density, accounts for the additional energy density to result in a flat Universe⁴. A third option is a tilted fluctuation spectrum, cold dark matter with $n \approx 0.7$. If n is significantly below 1, long-wavelength gravitational waves can be generated at the inflationary epoch. It has been argued⁵ that these gravitational waves, rather than density fluctuations, might be mostly responsible for the COBE fluctuations. All of these models preserve inflation and cold dark matter, in brave attempts to 'save the phenomenon'. To a sceptic, this might be reminiscent of adding epicycles to a ptolemaic universe, but the cosmologist uses the additional complexity to pose new challenges and predictions. Observations are the ultimate arbiter.

The Tenerife experiment is especially timely. The three frequency channels used — at 10, 15 and 33 GHz — allow discrimination between foreground emission, from our Galaxy, and the cosmic microwave background signal. There is an unambiguous detection of structure in the microwave background over the area of roughly 500 square degrees surveyed. The 5° resolution samples comoving scales (corresponding to the present epoch) of about 500 Mpc on the last scattering

IMAGE
UNAVAILABLE
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REASONS

The 10- and 15-GHz radiometers on Tenerife with Mount Teide in the background.

actual ripples in the cosmic background radiation. The signal was buried. Of course, it was statistically significant, and even confirmed by an independent balloon-borne experiment. A new experiment, however, performed at Tenerife and described by Hancock *et al.*² on page 333 of this issue, provides the first direct glimpse of individual features in the cosmic microwave background.

The detected structure is at a level of 2 parts in 10^5 on an angular scale of 5°. The scale of the features is too large to be compared to direct precursors of any known structure in the galaxy distribution. The sheer extent of the fluctuations means that no causal physical processes could have generated them since the Universe became transparent to radiation, about 300,000 years after the Big Bang. Rather, the microwave background fluctuations are part of a broad spectrum of fluctuations. Combination of the Tenerife data, at 5° resolution over a limited area, and the data from COBE, with resolution of 10° over the entire sky, sets strong limits on the possible shape of the primordial

fluctuation growth, first spelled out by the Soviet physicist Evgeny Lifshitz in 1946, requires primordial fluctuations to have been present. Following the discovery of the cosmic microwave background in 1964 by radioastronomers Arno Penzias and Robert Wilson, it was realized that these seed fluctuations should be visible on the cosmic photosphere, the last scattering surface of the cosmic microwave radiation that defines how far back we can directly peer into the remote depths of space.

The astronomers began a race that was to last 28 years to chase down the required fluctuations. As the observational limits improved, from 10 per cent in the original discovery paper, to one-hundredth of a per cent 20 years later, the predictions were refined, always remaining tantalizingly below the current limits. Inflationary cosmology intervened in 1981 to provide a physical origin for the fluctuations. Inflation predicted that the Universe is at critical density ($\Omega = 1$), and thereby helped to solidify the realization that the Universe was dominated by dark matter. The dark matter provided a gravitational

R.D. Davies

surface. The measured signal corresponds to fluctuations with a power spectrum normalization, assuming $n = 1$, of quadrupole amplitude $26 \pm 6 \mu\text{K}$. The COBE satellite team reported a value of $17 \pm 5 \mu\text{K}$. There is no inconsistency, although combination of the two data sets, as well as the second-year COBE data⁶, favours a value of n in excess of unity. A strong limit can now be set: $n > 0.9$. This suffices to destroy one of the suggested modifications to cold dark matter. Primordial gravitational waves of very long wavelength are not a dominant contributor to microwave background fluctuations.

It is too early to tell which of the remaining models will survive the onslaught of new data on degree scales now being obtained by at least half a dozen new experiments in locations that range from Saskatoon to the South Pole, as well as balloon-borne platforms. Foreground contamination from our Galaxy is a serious issue that continues to plague these experiments, all of which have very limited sky coverage, and may even affect the Tenerife result. The most intriguing possibility, if the Tenerife amplitude holds

up, is that the Universe is open, with $\Omega = 0.1-0.3$. Large-angular-scale anisotropies, at angular scales greater than the curvature scale of about $30\Omega^2$, tend to be suppressed by gravitational focusing⁷. This results in a systematic steepening of the radiation fluctuation strength on the largest angular scales. COBE measures anisotropies over $10-90^\circ$, and Tenerife probes $4-10^\circ$. At present the difference in amplitude of the anisotropy seen by the two experiments is only suggestive. Perhaps with improved measurements on these and still finer scales we will confirm that the curvature of the Universe really is imprinted on the sky. $\square$

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APOPTOSIS

Fascinating death factor

Suzanne Cory

LIFE requires death. Elimination of unwanted cells is vital for embryogenesis, metamorphosis and tissue turnover, as well as for the development and function of the immune system. The molecular dissection of cell death is proceeding apace, and last month there came yet another advance to think about — this is the identification, by Suda *et al.*¹, of the ligand which triggers cell death by binding to the cell-surface receptor variously known as Fas or Apo-1.

The Fas/Apo-1 story began in 1989, with the isolation of two monoclonal antibodies — anti-Fas (ref. 2) and anti-Apo-1 (ref. 3) — which had the startling property of killing a human cell line used as the immunogen. Cell death was rapid and took place by apoptosis, the suicidal process marked by cytoplasmic compaction, membrane blebbing, chromatin condensation and DNA fragmentation. Impressively, a single injection of antibody virtually eliminated a human B lymphoid tumour growing in nude mice³, raising intriguing prospects for cancer therapy.

Cloning revealed that the Fas and Apo-1 cell-surface antigens were one and the same^{4,5}. This transmembrane protein, of M_r 45K or so, is related to a family of receptors which includes the two tumour necrosis factor (TNF) receptors. Homolo-

gy between family members is usually restricted to the cysteine-rich extracellular domain, but the 55K TNF receptor I and Fas/Apo-1 also display homology (28 per cent identity) over an intracellular 'death domain' of some 80 amino acids^{6,7}. This domain lacks any recognizable signalling motif, so how it triggers apoptosis remains to be determined.

Interest in Fas/Apo-1 became more intense with the discovery that the gene is defective in mice that have mutations at the *lpr* (lymphoproliferation) locus⁸. The prototype *lpr* mutation prevents (or greatly reduces) receptor expression, while the *lpr*^{cg} point mutation within the 'death domain' inactivates receptor function. The inability of homozygous mutant mice to mediate Fas/Apo-1-induced apoptosis provokes a complex immunological disorder featuring defects in both the B and T lymphoid compartments⁹. The very similar phenotype of mice homozygous for the *gld* (generalized lymphoproliferative disease) mutation suggested that the *gld* gene encodes the ligand for Fas/Apo-1 (refs 9 and 10), a prediction that can now be tested.

Exploiting the observation that Ca^{2+} -independent killing by cytotoxic T cells operates through the Fas/Apo-1 pathway¹¹, Suda *et al.*¹ isolated the elusive Fas/Apo-1 ligand from a cytotoxic T hy-

bridoma by a sensitive expression cloning strategy. From its sequence, the Fas/Apo-1 ligand appears to be a glycosylated type II transmembrane protein. Hence receptor engagement probably usually requires cell-cell contact, although shed ligand may also prove to have a physiological role. The carboxy-terminal extracellular domain of the ligand bears striking homology to TNF- α and TNF- β . Like the TNFs, therefore, it is predicted to function as a trimer, activating the receptor by cross-linking.

The relationship between the death signals conveyed by Fas/Apo-1 and TNF receptors can now be scrutinized. Although the cloned rat Fas/Apo-1 ligand neither bound the human TNF I receptor nor activated the TNF receptor on a mouse lymphoma, the two receptors may associate functionally. It will be important to determine whether their 'death domains' interact with common or different intracellular proteins, and whether signalling affects the level of Bax or Bcl-x_s, recently discovered inhibitors of Bcl-2, the cytoplasmic protein which suppresses apoptosis induced by diverse cytotoxic agents¹².

The deadly Fas/Apo-1 pathway must be tightly controlled. It seems likely that both ligand and receptor expression are strictly regulated, and it is already clear that neither is readily detectable in quiescent mature lymphoid cells. Furthermore, receptor activity may be curtailed by labile inhibitors, accounting for the initial resistance of activated T cells to killing by anti-Fas/Apo-1 antibody, despite their high levels of receptor, and for the observation that certain cell types become susceptible only on inhibition of protein or RNA synthesis. Inhibition is unlikely to involve Bcl-2, because its over-expression provides only meagre protection against death induced through the Fas/Apo-1 (or TNF) pathway. Intriguingly, deletion of the carboxy-terminal 15 amino acids from Fas/Apo-1 increases its cytotoxic activity, suggesting that this region mediates inhibition⁶. Identification of the protein

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or proteins which bind to this domain might therefore uncover novel regulators of cell survival.

The physiological roles of this ligand and receptor remain enigmatic. The key to their immunological function surely lies in the unusual population of non-dividing T cells that enlarges the lymph nodes of *lpr* and *gld* mice. This disorder was initially attributed to a breakdown in the thymic purging of immature self-reactive T cells⁹. However, despite the expression of Fas/Apo-1 by most thymocytes¹³, it now appears that thymic selection remains intact in *lpr* and *gld* mice, and opinion is growing that their lymphadenopathy reflects the accumulation of terminally differentiated T cells. If so, a major role of Fas/Apo-1 may be to limit the amplitude and duration of the immune response.

Because Fas/Apo-1 is expressed in liver, ovary, heart and lung, it may regu-

late homeostasis or stress responses in these tissues. Indeed, wild-type (but not *lpr*) mice injected with an antimouse Fas monoclonal antibody died within eight hours, with wholesale destruction of the liver¹³. Clearly, increased understanding of the physiology of cell suicide is necessary before apoptosis can be harnessed for tumour therapy.

Finally, by analogy with the pleiotropic effects of TNF, the biological responses triggered by the Fas/Apo-1 ligand may not be confined to apoptosis. In certain cell types, or in combination with other signals, the Fas/Apo-1 receptor may in fact promote activation¹⁴ and thereby favour life over death. □

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PATTERN FORMATION

Spirals and chaos

J. P. Gollub

SPIRALS crop up as the dominant type of pattern in a remarkable variety of physical, chemical and biological systems. Recent developments include unexpected spiral patterns in thermal convection¹, and a possible explanation for the sudden onset of ventricular fibrillation². On page 345 of this issue, Assenheimer and Steinberg describe novel observations of spirals in thermal convection near the critical point of a fluid³. These examples show that studies of pattern formation in spatially extended nonlinear systems continue to reveal surprises, especially where chaos is involved.

Remarkable observations of nearly perfect spiral patterns in thermal convection were first made by Bodenschatz *et al.*¹ in 1991. Their work was based on measurements in high-pressure carbon dioxide gas, which allows systems very much larger than the basic scale of the pattern to be conveniently studied. Strikingly perfect hexagonal patterns also occur when the fluid properties vary significantly across the layer.

Last year, Morris, Bodenschatz and collaborators discovered a new form of spatiotemporal chaos composed of a disordered array of rotating spirals⁴. This work is noteworthy for its contribution to the problem of describing spatially extended chaos quantitatively, rather than qualitatively. On measuring the characteristic domain size or correlation length of the patterns, Morris *et al.* found a clear power law for the dependence of this length on the temperature difference measured from the convective onset. They were able to demonstrate that the

band of wavenumbers found in the spiral pattern lay almost entirely within the band for which stability theory predicts stable straight rolls. This apparent contradiction between theory and experiment is puzzling; the authors speculate that the straight roll pattern predicted by theory has a small basin of attraction, so that it is hard to reach from the initial and boundary conditions of a real experiment for temperature differences significantly above the onset of convection.

The work of Assenheimer and Steinberg in this issue explores patterns in Rayleigh-Bénard convection near the thermodynamic critical point, T_c , of SF₆. They take advantage of the fact that the fluid properties vary strongly with temperature near T_c , so that very thin layers only 130 μm thick can be used, and the two important fluid parameters can be easily varied. (These are the Prandtl number P , which is related to the viscosity, and a parameter Q that specifies how strongly the fluid properties vary across the fluid layer.) They add a new twist to previous work on spirals by showing that spiral patterns dominate for $P < 3.5$, whereas target patterns dominate for larger values. Furthermore, they are able to investigate the precise mechanisms by which one pattern is converted to the other through interaction with defects in the patterns.

The role of defects in non-equilibrium patterns is itself a challenge to work out. The situation is much like that in the physics of solids, where the dynamics of defects often controls the mechanical and thermal properties of crystalline matter. Many investigators have proposed that

the key to understanding non-equilibrium patterns lies in achieving a better understanding of defect nucleation, propagation and interaction. But this hope has been only partially realized (the interested reader is referred to a wide-ranging review by Cross and Hohenberg⁵).

How spatially disordered patterns or spatiotemporal chaos are generated is now one of the main themes of nonlinear science. Its possible importance is underlined by theoretical work² on the mechanism by which spiral patterns of excitation in cardiac tissue might become disorganized, possibly leading to fibrillation. Karma's work² is based on numerical simulations of the Noble model originally adapted from the classic Hodgkin-Huxley equations. The model has many of the properties seen experimentally in studies of cardiac tissue by imaging methods⁶. A particular oscillatory pulse instability of these equations can lead to the breakup of a large isolated spiral wave into a more complex pattern of many spirals with randomly distributed centres. The breakup of spiral waves has also been studied in other model systems relevant to excitable media. Of course, the adequacy of models based on a homogeneous medium remains to be established⁵.

Spirals are one of the possible basic elements of spatiotemporal chaos in non-equilibrium systems. By now a host of examples is known, with manifold variations on both the basic patterns and the transitional mechanisms. An adequate classification scheme does not yet exist, much less a general explanation of the phenomena.

Although the term 'chaos' is frequently heard in connection with deterministic nonlinear phenomena in extended systems, the standard methods of low-dimensional nonlinear analysis are very difficult to apply to extended systems. Worse still, the methods used for characterizing and distinguishing the different forms of spatiotemporal chaos seem inadequate. Although statistical methods are clearly needed, some of the more obvious candidates do not reveal the coherent structures (such as spirals and hexagons) that are visible even to the eye. This subject remains as murky as the turbulence it attempts to describe. □

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One gene — four syndromes

Veronica van Heyningen

WE generally expect one gene to be associated with one phenotype, but reports in this issue¹⁻³ (pages 375, 377 and 378) reveal mutations in the receptor tyrosine kinase gene *RET* that bring to four the tally of different phenotypes associated with constitutional mutations in this gene. Another paper⁴ (page 380) arrives with serendipitous timing for interspecies comparison. In this work, Schuchardt *et al.* used *ret*⁻ loss-of-function mutants, produced by targeted intragenic deletion, to examine the role of the gene in mouse development.

The *RET* gene was first defined as a dominant oncogene by classical DNA transformation assay. The oncogenic activation was shown to be due to genomic rearrangements in which the extracellular domain of RET (see figure) is replaced by amino-terminal protein fragments from various other genes. Such tumour-specific rearrangements, usually associated with abnormally sized messenger RNA, are frequently seen in papillary thyroid carcinomas⁵ (tumours of thyrocytes which do not normally express *RET*). Subsequently, high-level expression of normal-size *RET* mRNA was found in medullary thyroid carcinomas (calcitonin-secreting C-cell tumours) and in pheochromocytomas (adrenal chromaffin cell tumours)⁶. These tumours are usually sporadic, but familial cases are also known. In some, endocrine organs are affected simultaneously. Families can be classified according to phenotype into three different categories. First, familial medullary thyroid carcinoma (FMTC), which is the simplest and shows familial recurrence of tumours. Second, multiple endocrine neoplasia type 2A (MEN 2A), in which medullary thyroid carcinoma is often accompanied by pheochromocytoma and parathyroid hyperplasia (non-malignant overgrowth). And third, a more severe form, MEN 2B, which has all the features of MEN 2A, including very early tumour onset, and is also characterized by ganglioneuromas (neural cell hyperplasia) of the lips, tongue and colon, and sometimes skeletal and eye abnormalities.

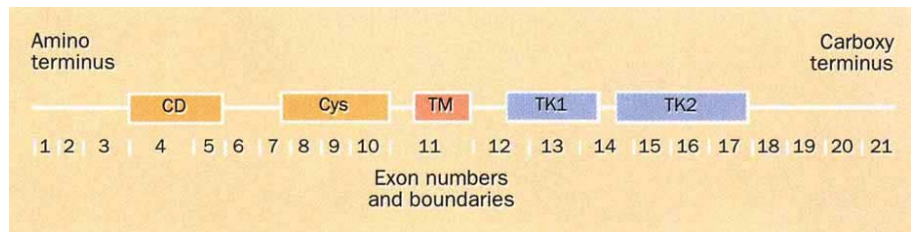
The *FMTC*, *MEN2A* and *MEN2B* genes segregate as incompletely penetrant dominant traits (offspring of affected individuals have a 50 per cent chance of inheriting the disease, but sometimes it can skip a generation). All three syndromes have been assigned by linkage to the chromosome 10q11.2 region⁷, where *RET* had also been mapped⁸. *RET* was therefore an obvious candidate gene for each of these familial cancer syndromes. Mutation analysis is still a rate-limiting

step in validating or discarding candidate genes suggested by disease mapping, but mutations were identified in MEN 2A and FMTC by two groups last year^{9,10}. Now, we finally have a report¹ (with Schuchardt *et al.*⁴ quoting another in press) of a qualitatively distinct mutation in several independent cases of MEN 2B and in a proportion of sporadic medullary thyroid carcinomas.

More surprisingly, however, there are also two reports^{2,3} of *RET* mutations

gene-knockout study in the mouse⁴.

The nature of the mutations is different in the thyroid-cancer-associated syndromes and in Hirschsprung's disease. The mendelian dominant inheritance is superficially the same in all four syndromes. But the Hirschsprung mutations include deletions and clearcut loss-of-function changes, such as premature stop codons, as well as some amino-acid changes which are assumed to lead to loss-of-function. The phenotype is probably explained by haplo-insufficiency (that is, sensitivity to 50 per cent loss of *RET* gene product in affected cells). In contrast, the cancer syndromes are associated with highly specific, amino-acid sub-



The *RET* gene encodes a receptor tyrosine kinase, and was first defined as an oncogene by classical transfection assay. Later it was shown that *RET* can undergo oncogenic activation *in vivo* and *in vitro* by cytogenetic rearrangement⁵. The predicted normal protein product from the 21-exon, 80-kilobase genomic gene has a cadherin-like (CD) ligand-binding extracellular domain with a cysteine-rich (Cys), calcium-binding region near the transmembrane region (TM), and intracellular tyrosine kinase domains (TK). Transcripts are of at least four different sizes, and their relative abundance varies from tissue to tissue¹².

in a completely different, often familial, developmental anomaly: this is Hirschsprung's disease, which affects about 1 in 6,000 people and is characterized by the congenital absence of parasympathetic innervation in the lower intestinal tract. Although the condition is now usually treatable, the most severe cases, with intractable constipation, abdominal distension and failure to thrive, often die. There are no reports of cancer-association with Hirschsprung's disease; rather, the idea of looking for *RET* mutations in patients suffering from the disease came about because family linkage mapping and deletion analysis had narrowed the position of the disease gene to the vicinity of *RET* (ref. 11).

So how can mutations at a single locus produce such clinically distinct phenotypes? And what is the connection between *RET* gene malfunction in thyroid malignancy and in the developmental malformations of Hirschsprung's disease and the *ret* knockout mice? The constitutional *RET* mutations described so far for the four distinct disease entities (FMTC, MEN 2A, MEN 2B and Hirschsprung's disease) are summarized in the table. The effects of the mutations can be considered using current understanding of RET function from its homologies (with other receptor tyrosine kinases and with cadherins) and from its expression pattern in mouse development¹¹, as well as from the

stitutions which are heterozygous in both normal and tumour tissue, suggesting that a single dominant hit at the *RET* locus is sufficient to initiate tumours. This is consistent with the observation that chromosome 10 allele loss is not seen in MEN 2-associated tumours, making them unique — in all other inherited predispositions to cancer (retinoblastoma and familial polyposis colon cancer, for example), homozygous loss of the relevant gene is required for tumorigenesis.

All nine of the MEN 2B cases and five of 18 sporadic MTCs examined by Hofstra *et al.*¹ have the same heterozygous missense change in the *RET* tyrosine kinase domain (see table). Reassuringly, the mutation is not seen in 70 normal controls, nor in the normal parents of one MEN 2B patient, confirming that we are not looking at neutral polymorphism. Like the MTCs, all the MEN 2B cases in this study are sporadic, but they have constitutional germ-line mutations. Cases of non-familial MEN 2B are frequent, presumably because when new mutations arise the developmental anomalies are often so severe that they preclude the sufferer from having children. The sporadic MTC mutations, observed in tumour tissue, were also heterozygous and presumed to be tumour-specific because of the absence of MEN 2B-associated developmental anomalies, although normal tissue was unavailable for analysis. It is suggested

RET mutations described to date

Disease	RET codon mutation (exon number) (f, familial; s, sporadic)	Number of independent cases
MEN 2A (ref. 9)	619Cys → Gly (10)	1
	635Cys → Arg (11)	12
	635Cys → Gly (11)	3
	635Cys → Tyr (11)	2
	635Cys → Ser (11)	1
	635Cys → Phe (11)	1
MEN 2A (ref. 10)	619Cys → Arg (10)	1
	619Cys → Ser (10)	2
	621Cys → Arg (10)	2
	621Cys → Tyr (10)	2
	635Cys → Arg (11)	1
FMTC (ref. 10)	612Cys → Trp (10)	1
	619Cys → Ser (10)	1
	619Cys → Tyr (10)	1
	621Cys → Arg (10)	1
MEN 2B – sporadic MTC – sporadic (ref. 1)	918Met → Thr (16)	9/9 5/18
Hirschsprung's disease (refs. 2, 11)	Cytogenetically visible deletions (s)	2
	Sub-microscopic deletion (f)	1
	373Ala del G 1120 (6) (f)	1
	765Ser → Pro (13) (s)	1
	897Arg → Gln (15) (s)	1
	972Arg → Gly (17) (f)	1
Hirschsprung's disease (ref. 3)	32Ser → Leu (2) (f)	1
	64Pro → Leu (2) (f)	1
	136Glu → STOP (3) (f)	1
	180Arg → STOP (3) (f)	1
	330Arg → Gln (5) (f)	1
	393Phe → Leu (6) (f)	1

that the methionine-to-threonine change is a dominant tyrosine kinase-activating mutation, perhaps causing altered target specificity, which would explain the hyperplasia leading to both tumours and ganglioneuromas.

The 28 independent *MEN2A* mutations^{9,10} (see table) all involve four conserved cysteines in the cysteine-rich region close to the transmembrane portion of the cadherin-like domain (see figure). These mutations probably interfere with ligand binding. Interestingly, the same mutations can elicit the milder FMTC phenotype. Romeo *et al.*² suggest that the *MEN2A* mutations are probably dominant negative, with the formation of a high proportion of non-functional dimers. I am more inclined to believe that the mutations in both *MEN2A* and *MEN2B* are dominant activating changes which lead to hyperplasia in cells that normally express RET. C-cell hyperplasia is invariably detectable before the onset of malignancy in known mutation-bearing MEN 2 individuals. Malignancy presumably arises in hyperproliferative cells through mutations in other genes. Ectopic

RET-activation by genomic rearrangement can cause malignancy in any tissue type.

How can the Hirschsprung's disease phenotype be reconciled with the normal role of RET and with loss-of-function mutations? The RET-expressing neural crest cells fail to migrate to their intestinal target site when RET dosage is reduced by half¹², perhaps implicating altered ligand-binding capacity. The severity of the phenotype varies even within families, and in many cases people with mutations show no phenotype^{3,4}. So cells do not always respond identically, even to the same mutation. Surprisingly, however, Hirschsprung patients show no phenotypic manifestations of the reduced RET expression in many other tissues derived from RET-expressing cells¹², such as cranial neural crest, part of the spinal cord, the developing kidney, the eye (eye phenotype is present in some MEN 2B patients, however), or the thyroid. This suggests differential dosage sensitivity in different cell types.

Finally, there is the question of how the human phenotypic spectrum compares

with that seen in mice. Mouse mutants created by *ret* gene knockout⁴ also exhibit the Hirschsprung phenotype to some extent, but with a number of important differences. Most strikingly, the knockout mice show no heterozygote phenotype. Homozygotes, however, are more severely affected than Hirschsprung patients. All *ret*⁻/*ret*⁻ mice die within 16–24 hours of birth — they have no intestinal parasympathetic neurons, and the kidneys are abnormal or absent⁴. This second component of the mouse phenotype is very interesting, not least because of its high variability which may be explained by stochastic processes in development or interactions with modifier genes, or both. The *ret*⁻/*ret*⁻ mice were produced on an outbred background, so selective breeding could reveal modifier genes.

Why are there no kidney abnormalities in Hirschsprung patients? Should we be looking for RET mutation in familial human kidney malformations? Differences in dosage-sensitivity between tissues have been a puzzling aspect for a number of heterozygous mouse and human mutations, and perhaps can be explained in part by tissue-specific splicing differences, such as those observed for RET (ref. 12). Functional redundancy, which may vary from tissue to tissue, is another possible answer.

The RET story illustrates many aspects of classical genetics, such as dosage effects and mode of inheritance. It shows, too, how efficient mutation analysis can illuminate molecular mechanisms and biological function. □

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Erratum

A mystifying 'w' appeared as a prefix to Ca²⁺ on two occasions in the figure accompanying Stuart A. Lipton's News and Views article of 13 January ("HIV displays its coat of arms" *Nature* **367**, 113; 1994). The symbol should have been a vertical arrow, denoting an increase in levels of intracellular Ca²⁺ as a consequence of neuronal excitation by toxins released from activated macrophages/microglia or other neurons.

Neutralizing acid rain

Evilie Gorham

ACID deposition is widely recognized as a regional environmental problem, the chief causes of which are oxides of sulphur and nitrogen emitted during fossil-fuel combustion and metal smelting. Sulphur dioxide emissions have been cut in recent years, but we have not seen matching declines in acidity of precipitation. Why the discrepancy? On page 351 of this issue, Hedin *et al.*¹ show that the deposition rate of atmospheric base cations (calcium, magnesium, potassium and sodium) has also fallen. Quite apart from its effect on acidity, the decline could adversely affect ecosystems that depend heavily on an atmospheric supply of these nutrient elements.

Acid rain was discovered in 1852 by Angus Smith, who later wrote a book on what he termed 'chemical climatology'², but his work sank into obscurity. Acid precipitation was rediscovered in the mid-1950s³ — as it happens, in three different regions. Houghton was studying cloud-water chemistry in New England. Barrett and Brodin were examining data from a network of Scandinavian stations set up to examine atmospheric inputs to agricultural ecosystems and expanded by Carl Gustav Rossby as a possible way of tracing the origins of air masses. Meanwhile, in the English Lake District I was testing the hypothesis that the ionic composition of pools on raised bogs must wholly depend on atmospheric precipitation (Einar DuRietz and Margareta Witting, whose hypothesis it was, had not actually analysed rain or snow).

Early studies (including Smith's) used a bulk collector set out continuously to catch both wet and dry deposition. A later system employed two buckets, with a cover that switched from the wet to the dry bucket during rain or snowfall. Unfortunately the dry bucket did not provide a satisfactory estimate of dry deposition, which remains very difficult to measure.

The chief impact of acid deposition upon ecosystems has been the acidification of streams and lakes bedded on rocks such as granites and quartzites, which are hard to weather and low in neutralizing bases. Aquatic floras and faunas are altered as waters acidify, often killing off fish stocks⁴. Deleterious effects upon terrestrial ecosystems are less clear, but may be substantial⁴.

Efforts to control acid deposition have focused on cutting emissions of sulphur dioxide, because sulphuric acid is a more important acidifying agent than are nitrogen oxides (once the latter have been converted to nitrate, they are rapidly absorbed by plants). Emission control has been effective, and sulphate deposition

has declined sharply over the past ten to twenty years¹, but without producing an equivalent reduction in precipitation acidity. In part, this could result from increasing emissions of nitrogen oxides, but Hedin *et al.* demonstrate that it is certainly a consequence of declining base-cation deposition.

Base cations in the atmosphere come from a range of sources^{5,6}. Ammonia, released in increasing amounts from manures and nitrogenous fertilizers, neut-

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REASONS

Acid rain — a clouded issue.

ralizes sulphuric acid in rain and snow. But the resulting ammonium sulphate becomes an acidifying agent once it reaches the soil, owing to exchange of ammonium for hydrogen ions during root uptake and to nitrification of ammonium ions by bacteria. Fly ash from urban and industrial smokestacks is often strongly alkaline. Particles of wind-borne soil can also be alkaline, especially on calcareous bedrock, and cement dust can neutralize acid deposition locally.

In a search for causes of declining base-cation deposition, Hedin *et al.* consider three possibilities. Of these, they find no evidence for a decrease in atmospheric precipitation that would wash base cations less effectively from the atmosphere. Nor do they observe a decrease in windy weather that could have led to fewer soil particles being entrained into the atmosphere. What they do find, from data compiled by government agencies, are strong declines in particulate emissions from regional urban and industrial 'point sources'. Emissions of base cations from non-point sources (such as unpaved roads) are more difficult to judge, but they too may have declined⁷.

The significance of a decline in base-

cation deposition is twofold¹. First, it lessens the influence of controls on sulphur-dioxide emissions in reducing acid deposition. Second, it reduces the supply of important nutrient elements to ecosystems, some of which rely on the atmospheric supply for growth. Although nitrogen and phosphorus are the most important nutrients limiting biomass accumulation, others may become significant, particularly as atmospheric deposition of nitrogen increases⁸.

What, then, should be done to follow up the new results? The monitoring of base-cation deposition should continue, and the other chief components of acid/base balance, ammonium and nitrate ions, should be incorporated if trends of acid (hydrogen ion) deposition are to be understood fully. Trends in the deposition of phosphorus, often a limiting nutrient, should be examined. Soil particles are rich in this element as well as in base cations⁶. If such particles decline, phosphorus will do so. Increasing use of phosphatic fertilizers may, however, increase wind-borne phosphorus and work in the opposite direction. The few results available suggest that urban and industrial particulates are about twice as rich in phosphorus as soil particles⁹, so any decline in these seems likely also to be important.

This all suggests that we need to determine the importance of wind-borne soil (including road dust) relative to urban and industrial particulates. Monitoring microscopically the types of atmospheric particulates deposited on the ground would be informative, and their very different trace-element signatures⁹ should let them be distinguished. Seasonal examination of deposition might also help, because wind-borne soil ought to be least abundant during snow cover and most abundant during spring cultivation.

The irony that reducing emissions of urban and industrial particulates lessens the efficiency of sulphur-dioxide control in mitigating acid deposition will not be lost on disappointed environmentalists, who will now have to call for yet more stringent regulation of sulphur dioxide. □

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High hopes for C4 plants

Peter D. Moore

THE so-called C4 photosynthetic mechanism equips plants with valuable competitive advantages under conditions of high light intensity, high temperature and low water availability. Their greater efficiency in trapping carbon dioxide means that they can exert a tighter control over water balance than most C3 plants, but plants endowed with the phosphoenolpyruvate (PEP) carboxylase initial carbon-fixing technique (which includes the crassulacean acid metabolism — CAM — plants) can also cope well with habitats in which CO₂ becomes scarce, as in the case of some aquatic environments in the middle of the day when planktonic photosynthesis is at its peak. It came as something of a surprise when CAM-type plants were first described from wet sites¹. But now another remarkable claim has been put

forward by V. S. Rama Das and S. K. Vats² of Palampur, India, who believe that C4 plants may be at an advantage at high altitudes because of lower levels of ambient atmospheric CO₂.

C4 plants operate a short-term mechanism for the fixation of CO₂ that involves the enzyme PEP carboxylase and results in the addition of the carbon from CO₂ onto a three-carbon receptor molecule, leading to a four-carbon product. This is then transported to specialized cells around the vascular bundles where the carbon is released once more and then permanently fixed by the conventional C3 system using the enzyme Rubisco. One important advantage of the C4 strategy is that PEP carboxylase has a higher affinity for CO₂ than Rubisco, so the C4 plant is able to maintain photosynthesis at lower

CO₂ concentrations (that is, it has a lower CO₂ compensation point).

It has long been evident that the C4 photosynthetic mechanism is more commonly found at lower rather than higher latitudes. It occurs in a wide range of plant families, both dicotyledons and monocotyledons, but was first described in, and is perhaps most familiarly associated with, tropical grasses. As one moves polewards, the proportion of C4 grasses among the species of this family gradually decreases, and only a very few grasses of the cooler temperate zone are C4 in character³. One such genus is the cord grasses (*Spartina*)⁴, which probably benefit from the water-relations advantages of the C4 mechanism (all species of this genus inhabit salt marshes, where salinity imposes physiological water stress).

The question of the competitive advantages or disadvantages of C4 photosynthesis under changed CO₂ conditions is currently topical because of the need to predict vegetation response to a green-

OBITUARY

Wolfgang Paul (1913–93)

ON 7 December Wolfgang Paul, who was awarded the Nobel Prize in Physics for his work in isolating ions and electrons, died at his home in Bonn.

Paul was born in 1913 in Lorenzkirch, a small town in Saxony. He studied physics in Munich and Berlin and obtained a doctorate in physics and engineering in Berlin in 1939. With his supervisor Hans Kopfermann he went to Kiel and then Göttingen. In 1952 he accepted the chair in experimental physics at the University of Bonn where he was director of the Physikalisches Institut until he retired in 1981.

Wolfgang Paul's broad interest in physics, his ingenuity and his experimental skills propelled the institute within a few years to international recognition in many areas of physics research. At all times he managed to inspire in his colleagues and students enthusiasm for new ideas and experiments. During his directorship he always led the institute according to the concept of 'guided anarchy', a term he coined himself, and which perhaps best illustrates his personal style of team research. Every student, graduate or even undergraduate, and every scientist of the institute had their own responsibility for a part of the experiment or accelerator, often in crucial areas. Of course, this did not always work ideally, and sometimes the topic of a student's thesis had to be changed at the last moment. Nevertheless, Paul kept to this concept of greatest possible freedom for the individual throughout his life. He believed this to be a key feature of university research and worked to integrate his concept into the cooperation

between research centres and universities.

In the field of atomic and molecular physics, Wolfgang Paul and his co-workers developed innovative methods for focusing and storing ions so as to allow precise

still in service as injector to the electron stretcher ring ELSA.

Wolfgang Paul was faithful to the University of Bonn despite distinguished offers from elsewhere. In particular, he was active in promoting fundamental research in particle physics in Germany. He was one of the founding fathers of CERN and DESY (the accelerator centre in Hamburg), acting as research director at both institutes at different points in his career.

In later years, as president of the Alexander von Humboldt Foundation, Paul directed his attention to the support of young scientists from all over the world. In particular, he shaped the Feodor-Lynen-Program for young German scientists, and consistently followed the principle 'quality over quantity' for which the Alexander von Humboldt Foundation is renowned.

After his retirement from the university, he maintained his commitment to individual scientific independence. The responsibility was now his successors'. When asked for advice, he would typically suggest "One could try to ...", but would always make it clear that the decision was to be made by someone else. As a result, his opinion was frequently sought and always much appreciated.

With Wolfgang Paul the University of Bonn loses an inspiring university teacher and an extraordinary scientist.

Norbert Wermes

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IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Wolfgang Paul, Nobel Prize winner in 1989.

measurements of their properties. For his achievements he was awarded the Nobel Prize in 1989, which he shared with Norman F. Ramsey of Harvard and Hans Dehmelt of the University of Washington. In the 1950s, the first European electron synchrotron to use the newly discovered principle of strong focusing was built under Paul's guidance in Bonn. The machine, which produced electron energies of 500 MeV, was completed in 1959, remained in use until 1985, and is to be reassembled for display at the Bonn branch of the German Museum for Science and Technology. In the 1960s a larger synchrotron of 2.5 GeV electron energy followed at the institute, and this is

house world⁵. But most experimental work, both in the laboratory and in the field, has concentrated on increases in ambient CO₂ rather than decreases. In the temperate zone, for example, will a higher CO₂ level reduce the competitive advantages currently enjoyed by *Spartina*? Will the balance of power shift in favour of its competitor C3 associates? An experiment has been performed⁶ in Chesapeake Bay, in the United States, where enclosed plots of vegetation were exposed to almost double ambient CO₂ levels (690 p.p.m.). *Spartina* was not significantly affected, but its main C3 competitor, *Scirpus olneyi*, benefited greatly and expanded its proportion of the vegetation biomass over the three years of the experiment. So, a greenhouse world may well shift the competitive balance of these photosynthetic strategies.

Findings of this nature, coupled with the discovery of CAM species in CO₂-depleted aquatic environments, makes it reasonable to speculate that habitats with low CO₂ concentrations may well offer competitive opportunities to C4 species, even in sites where water supply may not apparently be limiting. Such is the reasoning that Rama Das and Vats have applied to their observations in the Himalayas of north-west India. In conducting a survey of this region, which lies at an altitude of 1,300 m above mean sea level and enjoys a mild climate annually moistened by the monsoon, they found an unexpectedly high preponderance of C4 species among the grasses. In total, 79 per cent of those examined (both anatomically and for CO₂ compensation points) were classified as C4. Rama Das and Vats regard this high proportion as a reflection of the low CO₂ availability at high altitude giving a selective advantage to C4 species.

The claim, however, is not accompanied by data about levels of ambient CO₂, which is really required if it is to be justified. Some data are available for even higher altitudes in Alberta, Canada, however, where a recording station at an altitude of 2,100 m has a mean CO₂ level (at 10 m above the ground surface) very similar to that at a lower site (1,100 m) in

the same region⁷. The lower site has a greater diurnal and seasonal range of variation (probably because it is an agricultural site rather than a forest), but the means are close.

Recent discussions about the evolution and rise to prominence of C4 plants^{8–11} involve large variations in atmospheric CO₂. Those concerned generally regard 400–500 p.p.m. CO₂ as the requirement for efficient operation of the C3 system⁸ and 200 p.p.m. as stressful for plants of this type¹¹. It would appear, therefore, that the assumption that CO₂ becomes sufficiently scarce at high altitude to give C4 plants competitive advantage is not well founded, at least within the altitudinal range under discussion here.

How then can we account for the high preponderance of C4 grasses in a rela-

tively moist temperate environment? The Himalayas receive very high rainfall (2,500 mm annually), but even so drought may still be a critical factor as 65 per cent of this rain comes within the three-month period of the monsoon. Occasional acute drought, together with the high daytime temperatures typical of mountain habitats in the subtropics, may be enough to hand the advantage to C4 species. There is also the biogeographical question of what species are available for colonization, and therefore for competitive interaction, in this subtropical area isolated from the higher latitudes by the barriers of the high Himalayas. □

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MATERIALS SCIENCE

Polymers made to measure

Philip Ball

How long is a polymer molecule? The answer is usually the same as for the proverbial piece of string: any length you like. Or more properly, any one of a range of lengths, whether you like it or not. Most conventional methods of polymer synthesis produce polydisperse products, in which the chain length is poorly controlled. But at a recent meeting of materials scientists*, David Tirrell of the University of Massachusetts at Amherst explained how to make polymers in which not only is the molecular weight monodisperse — the chains have essentially the same length — but the stereochemical conformation too is well defined.

In much of materials science this kind of molecular precision is not necessary — only the bulk properties matter. But one message that emerged from the meeting was that, for the advanced applications that are likely to be required of materials in the coming years, they must be prepared to precise and reproducible molecular specifications.

If the synthesis of well defined polymers sounds familiar to molecular biologists, that is no coincidence. For Tirrell's group has exploited the ability of living systems to generate proteins in which the primary molecular structure is defined down to the last monomer and the three-dimensional shape is dictated by this structure.

The concept is, like most good ideas, simplicity itself (D. A. Tirrell, M. J. Fournier and T. L. Mason, *Mat. Res. Soc. Bull.* 23–28 (July 1991)). In short, Tirrell uses bacteria as polymer factories. The basic building blocks are inevitably amino

acids, but these provide no more than an architectural backbone and can be modified chemically either before or after assembly. The blueprint for the molecules is mapped out in synthetic genes — stretches of DNA prepared by solid-state organic synthesis. These stretches of synthetic DNA are incorporated into a suitable expression vector (a vehicle for protein synthesis) using methods that are routine in biotechnology, and inserted into bacterial cells. The microbe cultures then churn out the designed polypeptides to order.

In general, the target polymer contains several repeats of the synthetic DNA sequence, itself perhaps 50–100 nucleotides long. So the trick is to prepare and isolate multimers containing a specific number of these DNA monomers for incorporation into the expression vector. The monomers are synthesized initially with restriction sites at each end, which are recognized and snipped by a restriction enzyme after the monomers have been amplified. The monomers are then linked up to yield a distribution of multimers (see figure).

The easiest way to pull out from this population multimers of a specific size is first to insert them all into an intermediate vector, which can be screened according to the size of the insert. This procedure also allows the multimers to be capped with groups that will be needed for isolation of the final polypeptide — start and/or stop transcription signals, for instance, or cleavage sites if the polypeptide is to be expressed as part of a longer protein.

Finally the multimers with appropriate flanking sequences are cloned into the expression vector, typically a plasmid (a

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*Fall Meeting of the Materials Research Society, Boston, 29 November–3 December, 1993.

self-replicating circular strand of bacterial DNA) from *Escherichia coli*. The target polymer is then expressed *in vivo* and, if necessary, cleaved from its flanking sequences.

Tirrell's studies have so far focused primarily on two classes of polypeptide: sequences based on repeats of glycine-alanine, and glutamic acid polymers. One question that he has been able to address concerns the formation of polymer liquid crystal phases. Poly(benzyl glutamate)

this identification must await diffraction studies.

Incorporating more complex amino-acid sequences into these artificial polypeptides should allow the exploration of protein tertiary structure using model systems, and the design of polymer crystal structures using structural motifs familiar in proteins. Tirrell's group has built polymers containing glycine-alanine repeat segments (GlyAla) interrupted at every third step by glycine-glutamic acid units (GlyGlu). Strings of (GlyAla) are known to form β -sheets, while the hope was that the (GlyGlu) sections would serve to introduce turns, allowing the sheets to stack in smectic-like arrays. X-ray studies confirm that the polymer adopts the expected folded- β -sheet structure.

Chemically modified amino-acid residues might afford still more exotic materials. Tirrell described an example which might be dubbed a 'non-stick protein', containing fluorinated amino-acid residues. The idea was that the hydrophobic fluorine-containing groups might segregate from the interior to the surface of the folded polymer, engendering unusual surface properties.

The polymer is of the form $[(\text{Ala-Gly})_3\text{-F-Phe-Gly}]_x$, where F-Phe is a fluorinated form of phenylalanine. Once the artificial gene is incorporated into the bacterial cells, a strain must be developed that cannot make unfluorinated phenylalanine and can use the derivatized form in protein synthesis. Tirrell and

colleagues found good expression of the fluorinated polypeptide, with almost complete substitution of phenylalanine by the fluorinated variant. The material is rather insoluble in water, as one might expect; but its three-dimensional structure, and in particular the question of where the fluorine sits, remains to be resolved.

Particularly enticing from the perspective of materials design is the possibility of modifying structural proteins such as silk and collagen to improve their mechanical and chemical properties. Silk has long been hailed as a spectacular example of the superior properties of biomaterials; but the opportunity to improve on nature is always hard to resist.

Philip Ball is an assistant editor of Nature.

DAEDALUS

Dream no more

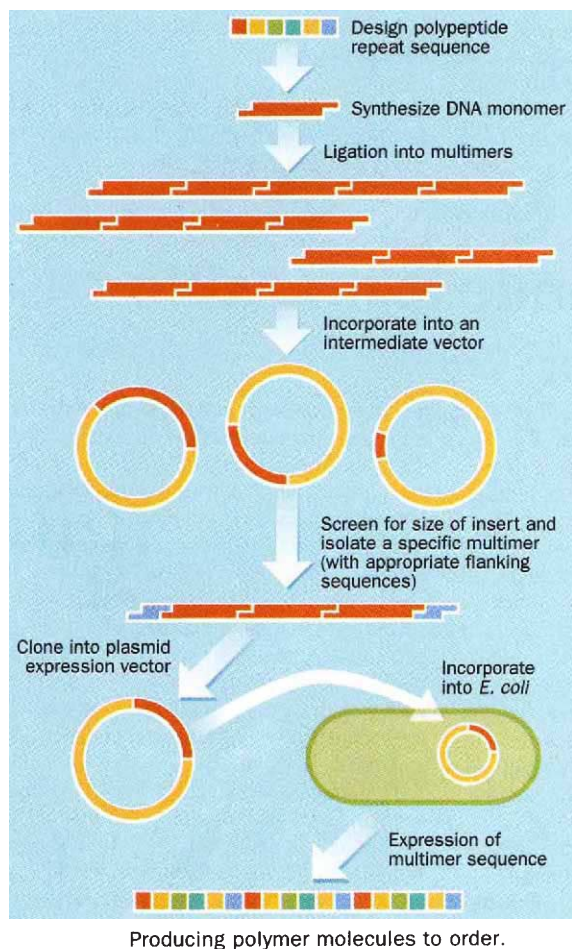
THE problem of free will, and the origin of mental inspiration, have long bothered philosophers. In the 1930s, it was suggested that the brain might amplify quantum uncertainty up to macroscopic levels. More recently a mechanism has been proposed: the firing of neurons by radiation. High-energy events in the brain, perhaps generating small bursts of neurotransmitter by some radiolysis reaction, fire neurons at random. Every so often, this 'noise' happens to trigger some critical neuron, one whose output avalanches into a whole new train of thought. This is the magical process of sudden whim or inspiration. External radiation is well blocked by the skull; most such events must come from decay of the brain's internal content of ^{14}C and ^{40}K . So Daedalus plans to remove these isotopes from the brains of volunteers, and observe the changes.

It will be a long job. Plants must be grown with ^{14}C -free CO_2 (from burning fossil fuel) as their sole carbon source. Their fertilizer must be depleted in ^{40}K by standard isotope separation methods. When harvested, they will form a rigorously radiation-free vegetarian diet. Volunteers fed exclusively on this diet will, by the steady metabolic turnover of all their tissues, slowly come to be radiation free themselves.

Deprived of inspiration, but with their memory and reasoning power still intact, Daedalus's volunteers will inhabit a very ordered mental world. Calmly logical in a crisis, they will be highly predictable in routine conditions. A radiation-free diet may help hyperactive children and butterfly-mind geniuses to complete the tasks they begin. In psychiatry it may quench the insane whims of psychotics and the chaotic enterprise of manics: but obsessives, of course, would become more obsessive than ever. The diet might even replace gaol for impulsive criminals. Deprived of random temptation, they might abandon crime: if not, they would be predictable enough to catch easily. On giving up the diet, a user would slowly take up natural levels of radioisotopes and, over several months, recover his previous personality.

An isotope-enriched diet should have the converse effect. Scientists whose inspiration has failed them and novelists paralysed by writer's block might be revitalised by a diet carefully elevated in ^{14}C and ^{40}K . Daedalus wonders if the physicists of the heroic age owed their towering creativity to the radioactivity they studied so carelessly. If so, modern physicists yawning over yet another plodding, uninspired theory or experiment can blame today's safety regulations.

David Jones



(PBG) is known to form at least one nematic phase, in which the chains exhibit orientational but not positional order. Small-molecule nematic liquid crystals often undergo a transition to a smectic phase as temperature is lowered, in which the molecules are arrayed in layers with a regular repeat distance perpendicular to the layers. But the polydispersity in chain length of regular PBG may be expected to inhibit the formation of a smectic phase.

To address the issue of whether monodisperse PBG can form a smectic phase, Tirrell prepared poly(glutamic acid) via his microbial factories and then used standard synthetic methods to derivatize the chains to PBG. The resulting compound does indeed show a texture, under the polarizing microscope, that is suggestive of a smectic phase. But confirmation of

Satellite greenhouse signal

SIR — The temperature of the global atmosphere, particularly the lower troposphere (the lowest 7 km), is expected to rise because concentrations of gases such as carbon dioxide are increasing. Sophisticated yet still relatively idealized ocean-atmosphere models indicate that the temperature of this layer will rise at a rate of 0.3 to 0.4 °C per decade^{1,2}. This layer has been monitored since January 1979 by microwave sounding units (MSUs) on polar orbiting satellites, providing perhaps the best measurement of the Earth's air temperature in terms of its global coverage and precision³. The length of this satellite record, however, is relatively short for determining climate trends.

The unadjusted 15-year global MSU data (T_{2R}) reveal a trend not significantly different from zero (curve *a* in the figure). Herein lies the issue at hand. In this period, as greenhouse gases have increased rapidly and given that their accumulated effects should be quite apparent by now, why is there no observed warming?

The answer, apparently, is that there were temperature-related events other than enhanced greenhouse warming in the atmosphere during this period. Two large volcanic eruptions, El Chichón in 1982 and Mount Pinatubo in 1991, have caused periods of reduced solar radiation, and therefore cooling⁴. Four El Niño–Southern Oscillation (ENSO) events have also been observed. Our goal is to calculate and then remove these ENSO and volcanic effects from the measurements and to examine what remains.

The ENSO signal on T_{2R} is taken from the sea surface temperatures (SSTs) of two tropical Pacific regions, Niño 3 and 4 (refs 5–7). A simple linear regression of the SSTs in these regions against the global T_{2R} explained the most variance (69.7%) when the atmosphere lagged behind the SSTs by 5 months. A 5.5-year period (July 1982 to December 1987) was the best for calibrating the influence of ENSO on T_{2R} ; further testing showed no dependence between the effects of El Chichón and the 1982–83 ENSO. This ENSO effect is shown in curve *b*.

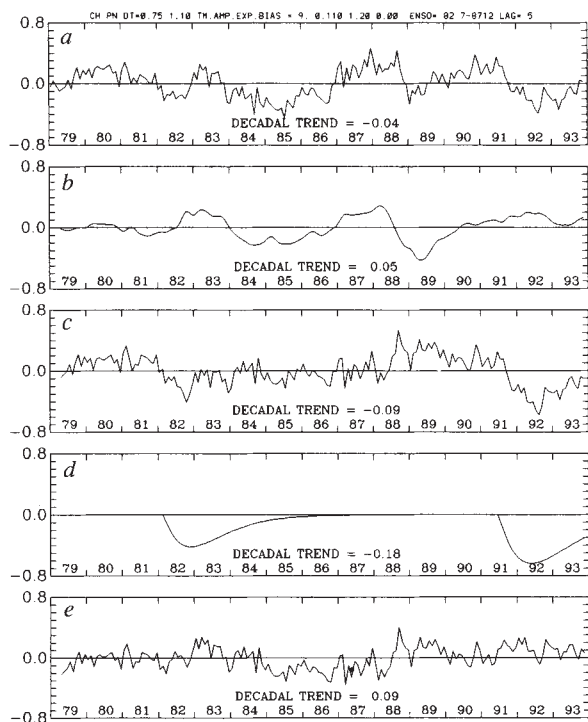
Sulphur compounds are often injected into the strato-

sphere during volcanic eruptions and, when in contact with water, may become aerosol particles of sulphuric acid. These aerosols can scatter sunlight back to space, resulting in less solar energy reaching the lower atmosphere and therefore cooling it^{4,8}. These same aerosols also trap thermal energy coming from below, leading to a warming of the stratospheric air. The warming in the MSU stratospheric temperature (MSU 4 or T_4) after the eruptions, then, can represent the magnitude of the volcanic shading effect⁹. We found the temperature increases (ΔT_4) to be 0.75 and 1.10 °C for El Chichón and Mount Pinatubo, respectively. We devised a formula in which the ΔT_4 predicts the magnitude and duration of the T_{2R} cooling

$$T_{2R,VOL} = \alpha j^\gamma \exp(-j/\tau),$$

where $\alpha = \alpha_0 (\Delta T_4)^{0.5}$; j is months since eruption; $\gamma = \gamma_0$; and $\tau = \tau_0 (\Delta T_4)^{0.5}$.

We selected the coefficients α_0 , τ_0 and γ_0 (0.11, 9.0 and 1.20, respectively) to fit the observations (curve *c*) of the volcanic impact on T_{2R} . This effect is shown in *d* and the resultant global temperature, once ENSO and volcanoes are removed, is shown in *e*. There was little flexibility in selecting the coefficients because of the fitting requirements. Other simulations for *e* all gave trends within the 0.08–0.09 °C range.



a, Monthly global anomalies of T_{2R} (relative to the base period 1982–91) to November 1993 (°C); *b*, ENSO effect from SSTs; *c*, curve *a* minus *b*; *d*, volcanic effect; *e*, residual time series after ENSO and volcanic effects removed.

Curve *e* reveals an upward trend of +0.09 °C per decade, or about one-quarter of the magnitude of climate model results. Although this residual trend could be greenhouse warming, other factors whose effects are less quantifiable (for example cooling via tropospheric aerosols or fluctuations resulting from solar variations) may also be involved.

Observational detection of the enhanced greenhouse signal is critical in evaluating how the Earth system reacts to increases in infrared-active trace gases, and how well climate models portray the atmosphere. Because of its global coverage, precision and measurement layer (where the global signal should be largest), the MSU temperature record perhaps offers the best opportunity for finding the greenhouse signal in a single variable.

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Weekdays warmer than weekends?

SIR — Microwave sounding units (MSUs) on NOAA orbiting satellites have been recording atmospheric temperature data for more than 14 years. The data have been merged into a homogeneous set by Spencer and Christy (see refs 1,2 for a technical description, analysis and evaluation of the instrumentation and data calibration). Here I am concerned with the data for the lower troposphere, that is, for the layer 1,000–400 hPa. This layer may be regarded as fairly representative of the troposphere as a whole and may be a more accurate guide to hemisphere and global temperature trends than have been computed from historical time series of mean temperature anomalies. The results to the end of June 1993 indicate that the overall global trend is slightly negative, more so in the Southern than in the Northern Hemisphere. This may be due in large part to the Mount Pinatubo volcanic eruption.

It is generally recognized³ that it is not possible unequivocally to detect any global warming that might be caused specifi-

MEAN TEMPERATURE OF LOWER TROPOSPHERE FOR DAYS OF THE WEEK

	Globe		NH		SH	
	Temp.	s.d.	Temp.	s.d.	Temp.	s.d.
Sunday	-4.95	0.2276	-7.71	0.2856	-2.37	0.2595
Monday	-2.57	0.2254	-2.88	0.2847	-2.38	0.2611
Tuesday	1.13	0.2273	3.54	0.2838	-1.30	0.2580
Wednesday	6.14	0.2253	11.34	0.2789	1.15	0.2593
Thursday	0.65	0.2234	1.38	0.2749	.18	0.2607
Friday	2.56	0.2224	.08	0.2767	5.04	0.2607
Saturday	-2.84	0.2214	-5.88	0.2788	.06	0.2551

Temperature in °C and normalized $\times 1,000$; s.e. of daily values is 0.06 °C s.d. in °C.

cally by an enhanced greenhouse effect. But it might be possible to detect a heat signal from human-induced activities according to the days of the week because industrial activity throughout much of the world eases to some extent during weekends. In addition, commuter automobile traffic is also reduced (despite some increase in recreational driving). The 5-day working week exists in many western industrial countries, and Friday is a holiday in the Muslim world.

I have therefore tabulated daily satellite temperature anomalies for the 7 days of the week. The series stretched from 1 January 1979 (a Monday) to 30 November 1992, a total of 5,083 days. Neglecting the final day, the series composed 726 weeks. There were a few missing observations, but not many. The latter intervening missing observations days were allocated linearly extrapolated values, and the results are shown in the figure and table.

The broad sine-curve variation for the days of the week exhibited by the North-

ern Hemisphere is quite remarkable: the anomalies agree with the hypothesis that a human-induced heat signal is present. The puzzling peak on Wednesdays could be explained by the fact that the new weekday in the dataset occurs at noon at Greenwich (Universal Time). Thus at

midday on Thursday, Friday is already

starting to move over the eastern half of

the world. This argument favours a possi-

ble Wednesday maximum in the Northern

Hemisphere, where the Muslim faith is

more predominant east of the Greenwich

meridian. A further argument to explain

the Wednesday peak could be that various

holidays in the Western world tend to

congregate between Wednesday and

Tuesday. For example at least 2% of

Thursdays are Thanksgiving holidays in

the United States, while the effect of

Fridays is even more pronounced.

The variation in the Southern Hemis-

phere, which appears to lag about 2 days

after the Northern Hemisphere, is less

clearly defined. The difference may re-

fect the different land and ocean propor-

tions, as there is approximately twice as

much land in the Northern than the South-

ern Hemisphere, and so the proportions

of human induced heat activity would be

much greater in the North. A more de-

tailed analysis of land versus ocean cover-

age is not yet possible on the

time scale of a day with the

MSU satellite set.

A one-tailed *t*-test is

appropriate as the predic-

tion is that the warm peak

should occur mid-week and

the cold minimum at the

week end. The *t*-test then

indicates that the difference

in the means between Sun-

day and Wednesday in the

Northern Hemisphere

curve is significant to the

10% level. Although the

significance does not reach

the orthodox 5% level, it is

meaningful. A sign test of

the differences between

Wednesdays and Sundays indicates better

than 5% significance. The Southern

Hemisphere curve is not significant to any

acceptable level. Although the amplitude

of the curve is only about 0.01 K, the rate

of change of 0.02 K in 3.5 days is 70 times

greater than the predicted enhanced

greenhouse trend of 0.3 K per decade.

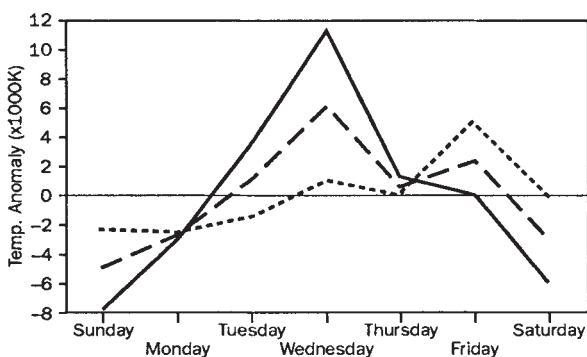
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Mean temperature anomalies for the lower troposphere. Solid line, Northern Hemisphere; dotted line, Southern Hemisphere; dashed line, globe.

ern Hemisphere is quite remarkable: the anomalies agree with the hypothesis that a human-induced heat signal is present. The puzzling peak on Wednesdays could be explained by the fact that the new weekday in the dataset occurs at noon at Greenwich (Universal Time). Thus at

Wednesdays and Sundays indicates better than 5% significance. The Southern Hemisphere curve is not significant to any acceptable level. Although the amplitude of the curve is only about 0.01 K, the rate of change of 0.02 K in 3.5 days is 70 times greater than the predicted enhanced greenhouse trend of 0.3 K per decade.

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Hotspots and species diversity

SIR — Using data from the British Isles for several groups of organisms, Prendergast *et al.*¹ found only “weak support” for the principle that conserving areas of highest species diversity (‘hotspots’) is the best way to conserve rare species. For one of their groups, birds, we are able to test their conclusions using published, coarse-resolution data on the terrestrial birds of Australia², a landmass that is larger, much less populated, and contains a greater diversity of habitats than Britain. Although we find some similarities, there are important differences between the results of the two studies.

Prendergast *et al.* used a spatial resolution of 10-km squares and defined rare species as those occupying <16 out of 2761 squares (32 of 206 species). We used data with a resolution of 1°×1° (~100-km squares) and a proportionally similar definition of rare Australian species: those occupying <5 out of 812 squares (30 of 433 species). In both studies, hotspots were the top 5% of squares ranked by total number of species.

Like Prendergast *et al.*, we find that about half of rare bird species do not occur in hotspots (54.4% in Australia and 43% in Britain). But in contrast, we find that 47% of Australian hotspots contain at least one rare bird species, compared to only 22% of British hotspots. Only one rare Australian bird species is located in a ‘coldspot’, compared to 19% of rare British birds.

These similarities and differences support two suggestions of Prendergast *et al.*, that their results are both scale-dependent and specific to fragmented landscapes (such as the British Isles). At progressively coarser scales of resolution, rare species and hotspots will tend to be coincident because sampling squares will ‘need’ rare species to become unusually species-rich. If landscapes have different levels of fragmentation, then greater fragmentation can leave the rare species stranded capriciously in widely scattered areas, each containing relatively few species. Britain is much more fragmented than Australia, which could explain our second result that rare species contribute more to Australian hotspots than to British ones. Most British hotspots are apparently composed of many relatively common species, whereas half of Australian hotspots contain some rare species, and a few are virtually defined by rare species (areas of high endemism). Finally, Australian coldspots are likely to have much lower species diversity ▶

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than those in Britain, resulting in relative absence of rare species.

We agree that species diversity should not be the sole criterion for identifying areas for conservation, but our evidence suggests that the relationship between diversity and rarity is dependent on both the fragmentation of the landscape and the scale under consideration. Discovering the exact nature of this relationship is

of considerable interest for conservation purposes. Targeting areas of high diversity may be the best way to protect rare species only if very large areas are available for conservation.

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Tool use by wild chimpanzees

SIR — Wild chimpanzees at Bossou, in the Republic of Guinea, West Africa, extract sap from oil-palm trees with a “pestle”, and then drink it using a “fibre-sponge”. This behaviour includes the use of techniques similar to those used when wild chimpanzees use other tools, such as the stone hammer-and-anvil^{1,2}, the digging-stick^{3,4}, and the leaf-sponge⁵. However, both squeezing the sap and the use of the pestle have not, to my knowledge, been reported previously (see ref. 6).

In the periphery of the core-area of a wild chimpanzee group at Bossou Oil-palm trees (*Elaeis guineensis*) are frequently found. A mature tree is more than 20 m in height and its top is covered with 20–40 large pinnate compound leaves. A hard leaf-stalk (petiole) reaches 5 m in length and 5–10 cm in breadth. All resident members of the chimpanzee group have been identified since 1976; the group consisted of about 20 chimpanzees throughout the study period.

Chimpanzees come to feed on oil-palm nuts using hammer-and-anvil stones. Some of them climb on the top of the tree, stand on two legs, grasp an upright young leaf-stalk in the centre of the crown with both hands and pull it out from the tree with great force. Even for an adult chimpanzee it takes more than a minute to detach it. After taking off a leaf-stalk from the tree he or she bites its white base and sucks sap from it (a in the figure).

On 7 January 1990, in the driest season, after taking and throwing away more than 5 young stalks, an old female of about 40 years bit off thorns of a stalk with her incisors, stood up on two legs at the edge of the tree-top and pounded its centre

vertically with the stalk, as if she were pounding grain with a mortar and pestle. After 10 poundings with both hands and with much force, a hole appeared in the centre of the tree-top. The chimpanzee put her left hand in the hole and picked up a handful of drenched fibre which she had made by the repeated poundings. She sucked it as she would a sponge. She dropped it into the hole, brought it up and sucked again. She also licked her drenched hand. After she climbed down the

tree an adolescent male climbed up. He took off a leaf-stalk from the tree and did the same as the old female (b in the figure). He pounded 10 times with both hands and 6 times with his right hand. He squatted and put his left hand into the deep hole which must have been more than 30 cm in depth as his elbow was completely in the hole. He repeatedly brought up a lump of fibre from the bottom of the hole and sucked sap from it.

Many adult and adolescent chimpanzees at Bossou were seen every dry season to pull out palm leaf-stalks; however, the combined work of the “pestle pounding” and “fibre-sponge sucking” was confirmed for only four episodes in two adult females (estimated 30 and 40 years old) and two adolescents (9-years old male and 8-years-old female).

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a, Adolescent chimpanzee biting and sucking sap from the base of a young leaf-stalk. b, Adolescent chimpanzee pounding the hole at the top of a palm tree with a leaf-stalk as the “pestle”.

Sex-ratio and inheritance

SIR — The theoretical implications of the reported inheritance of acquired characters in Mongolian gerbils¹ are so important that the evidence should be assessed most critically.

Clark *et al.* report¹ that adult female gerbils who gestated between two male fetuses (2M females) produce litters with a significantly greater proportion of sons than those (2F) mothers who gestated between females. Gestation of females adjacent to males causes their androgenization. If the report is valid, female offspring of 2M mothers have an enhanced chance of also being 2M, and the masculinized phenotype will tend to be perpetuated.

But Clark *et al.* do not report the significance of departure of the sex ratio from 50% in litters born to 2M mothers; the excess of males in these litters (57.1%) was similar to their deficit in those of 2F (43.7%). The androgenization theory they proposed provides no explanation for the latter figure.

Clark *et al.* have elsewhere shown² that prepartum gerbil litters overall contain equal numbers of the sexes, irrespective of litter size, but that litter size correlates with sex-biased perinatal losses. They suggest that mothers of small litters selectively cannibalize their own new-born

daughters, while mothers of large litters selectively cannibalize their sons. On average, 2M females mature later than 2F, have rather smaller litters and differ behaviourally in several respects^{3,4}. Could the reported differences in sex ratios within day-old litters born to 2M and 2F mothers be due to sex-biased cannibalization of female pups by 2M and of male pups by 2F mothers?

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CLARK AND GALEF REPLY — Pritchard notes first, that androgenization of female gerbils gestated between males does not provide a sufficient mechanistic explanation of the phenomenon under discussion and, second, that differences in the sex-bias of maternal cannibalism directed towards large and small litters might explain the observed result. We agree with Pritchard that androgenization of females fails to provide an adequate causal explanation of the effects of intrauterine position on litter sex ratios. We do not, however, believe that our data are consistent with Pritchard's suggestion that sex-biased cannibalism directed towards litters of different sizes can explain our results¹.

Pritchard errs in stating that litters delivered by dams that gestated between two male fetuses (2M females) are rather smaller than are litters delivered by dams that gestated between two female fetuses (2F females). In the paper he cites³, the difference in mean size of litters delivered vaginally by 32 early- and 36 late-maturing gerbils was not statistically significant. In the 50 litters born to 2M and 2F females studied in ref. 1, the mean size ($\pm$ s.e.m.) of litters delivered by 2M females (6.3 ± 0.3 pups) did not differ reliably from the mean size of litters delivered by 2F females (6.7 ± 0.5 pups; Student's *t* test, *t*(48) = 0.68, NS). It is difficult to see how unreliable differences in litter size could cause reliable differences in litter sex ratio.

To address the more general issue of effects of perinatal mortality on the sex ratios of vaginally delivered gerbil litters, we have compared the sex ratios of caesarian-delivered litters of 2M and 2F female gerbils. To date, caesarian-delivered litters of 2M female gerbils have contained a significantly greater percentage of male fetuses than have caesarian-delivered litters of 2F female gerbils (Mann-Whitney *U* test, *U* = 0, *P* = 0.05). Although our current sample sizes are

small (*N* = three per group), available evidence is not consistent with the hypothesis than any form of sex-biased perinatal mortality is responsible for differences in the sex-ratios of litters delivered vaginally by gerbils gestated in different intrauterine positions.

Because of the scarcity of 2M and 2F females in the relatively small litters produced by Mongolian gerbils, it will be several months before we can complete our examination of sex ratios of caesarian-delivered gerbil litters from 2M and 2F females. However, Vanderbergh and Huggett (personal communications, 19 November 1993) find a highly significant correlation (*P* < 0.002) between the intrauterine positions in which mouse dams (*Mus musculus*) gestate and the sex ratios of their litters. The far greater frequency of 2F and 2M female fetuses in the large litters of mice than in the small litters of gerbils should permit rapid progress in determining the causes of intrauterine-position effects on the sex ratios of rodent litters.

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Bayesian quantum mechanics

SIR — Maddox¹ writes of "what many people have been driving at for the past decade, the idea that the probability aspects of quantum mechanics should be built into the foundations of the subject." Wiener and Siegel² were early proponents of such a programme. Bohm³ characterized their ideas as implying "a change of possible structures that would perhaps be as great as that implied by the change from ptolemaic epicycles to newtonian equations of motion."

The differential-space theory used by Wiener and Siegel has been the basis for further work⁴⁻⁶. As a consequence of these subsequent analyses, one can assert (speaking in terms of the finite-dimensional case) that an $n \times n$ density matrix (ρ) of a quantum system is, in effect, the covariance matrix of a complex multivariate normal (gaussian) distribution over the vectors (wavefunctions) of n -dimensional complex Hilbert space. (This can, equivalently, be thought of as a covariance matrix in $2n$ -dimensional real space, with a specific form of structure^{7,8}. The mean vectors in both the real and complex cases are null.)

There have been few applications of Bayes's theorem⁹ in quantum mechanics¹⁰ — though it has been used to prove Bell's theorem¹¹. The basic impediment has been the apparent lack of suitable prior

distributions over the pure and mixed states of quantum systems¹⁰. However — relying upon the multinormal interpretation of ρ — one can use as prior distributions those that have been developed for this class of distributions.

In the bayesian inference of the covariance matrix of a multinormal distribution¹², one can, in the limiting case of minimal prior information¹³, attach a vague, Jeffreys, invariant prior, proportional to $|\rho|^{2n+1}$, to ρ (ref. 14). Pure states, for which $|\rho| = 0$, are, thus, assigned null prior probability. In the simplest ($n = 2$, spin-1/2) case, the improper prior $|\rho|^5$ has been normalized¹⁴, so that its integral over the pure and mixed states is unity. This normalized form is $9,009 (1 - r^2)^5 / 1,024 \pi$, where r is the distance of a state from the centre in the (Riemann sphere) representation of spin-1/2 states by the unit ball in three-space. Normalization is more problematical for $n > 2$.

Jones¹⁰ had, in fact, applied Bayes's theorem to the problem of quantum state estimation/determination. However, prior distributions that were simply uniform (unitarily invariant) on the pure states, and null on the mixed states were used. Jones did make and rely on the critical observation that eigenstates onto which measurements project should be regarded as the realizations of the underlying stochastic process. Such realizations could be used to form a sample covariance matrix, from which one could obtain the likelihood and then, by Bayes's theorem, the posterior distribution (the product of the likelihood and the [multinormal] prior $\propto |\rho|^{2n+1}$) (refs 9,11,12).

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references.

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Standing in line

Graham Ritchie

From Carnac to Callanish: The Prehistoric Stone Rows and Avenues of Britain, Ireland and Brittany. By Aubrey Burl. Yale University Press: 1993. Pp. 286. £25, \$45.

THE standing stones of Britain, Ireland and Brittany capture the imagination of those who live daily in their shadow as well as those who chance upon them as tourists and travellers. The folklore associated with stones is as varied for single monoliths as it is for stone circles and linear arrangements. Circles and rows have a greater potential for drama than do the single uprights, epitomized by the legend of the Breton wedding party that refused to let a priest pass and was punished by petrification. One stone on the island of Jura has been likened to Queen Victoria in repose, and no-one looking at plate 15 of Long Meg and her Daughters near Penrith in Aubrey Burl's handsome volume can doubt that stones can become anthropomorphized very readily. It all depends on imagination.

Burl's *The Stone Circles of the British Isles* (Yale University Press, 1976) has rightly become a classic of archaeological presentation, outlining different types of monument and making an assessment of their dating evidence and social context. In the intervening years he has continued the study and has not avoided discussion of such controversial issues as complex geometrical shapes and the potential significance of astronomical alignments in the positioning of sites within their landscape. The present volume takes a more disparate group of monuments, linear settings of upright stones, stones in rows and avenues, and creates order where none had existed before. Much archaeological writing depends on giving the reader information about a group of individual monuments, location, earlier records of excavation and the surviving evidence, and then drawing the threads together in an interesting way; Burl is particularly skilful at doing this, incorporating engaging writing with unexpected allusions, thus maintaining the reader's interest throughout. Many of Burl's own fine photographs illustrate the book and help to evoke further the topographical setting of these monuments. Dating evidence is sparse, for it is rare that the hole in which a monolith has been set up has been excavated to modern standards and even rarer that useful dating evidence

has been discovered. In some cases a burial deposit has been found set into the stone-hole, but in most cases excavations around such sites have provided only the broadest indications of age. Thus Burl's classification makes the best use of a comparatively small amount of evidence and relies primarily on the number and arrangement of the stones in individual settings.

Burl believes that long stone rows developed from the avenues that led to stone



Stones in Co. Cork, possibly aligned on the southern moonset.

circles such as those at Avebury and Callanish, and he lists other less well-known examples. The archaeology of such monuments is fully discussed, with both the possibilities and the problems fairly stated. The type of social structure capable of planning and accomplishing such engineering feats is evoked both by the surviving artefacts and by an assessment of the man-hours that might have been required. Such estimates are notoriously difficult to make, but a modern parallel involved 170 people pulling a 31-ton block at no more than 100 yards a day. Stone rows not associated with any circle are a particular feature of Exmoor and Dartmoor, although a recent resurvey on Learable Hill in Sutherland confirms this as a northern outlier. Burl's photographs give a good impression of the scale of the rows in terms of their length within the land-

scape, and the experience of survey, even with present-day equipment, underlines respect for the conceptual and constructional skills involved.

Rows of stones have not before been subjected to rigorous classification, and Burl's study is of particular value in drawing together a range of material from Ireland, Britain and Brittany and organizing it into a number of distinct categories: single rows of stones, multiple rows of stones, sometimes parallel sometimes in fan-like settings, and shorter rows in recognizable groups of between four and six, three, and pairs of stones. Although regional studies of such sites have been available in the past, Burl's volume is the first to place such sites into a broader context. Although the multiple rows capture the imagination of visitors to Brittany, both because of their vastness of

scale in linear terms and because of the size of the stones, the fan-shaped settings of northern Scotland are equally complex in layout. Burl rightly draws attention to the importance of preserving the sites and aspects of their topographical setting for future research.

Shorter linear settings have puzzled archaeologists for generations; they are difficult to describe in such a way as to make a satisfactory evocation of the monument as an impressive statement in a prehistoric landscape. Yet they are major monuments of antiquity. Burl's impressive photographs do full justice both to the enigma of the stones themselves and to the topography in which they stand, perhaps as the foci for funerary or social rituals. Dance has always seemed a likely part of rituals using formalized layouts of stones, but it is impossible to document

archaeologically.

The apparatus of notes, county concordance and gazetteers is comprehensive, but quite difficult to use: Welsh counties are in their modern form, whereas Scotland is in its pre-1975 counties, but with the islands of Argyll scattered alphabetically. The constant user of the volume will come to grips with the mass of information; the more general reader will have no need of such background material and will be delighted by a lucid and well presented account of a comparatively unexplored aspect of prehistory. □

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Seminal discussions

Keith Dudley

Molecular Biology of the Male Reproductive System. Edited by David de Kretser. Academic Press: 1993. Pp. 483. £76, \$99.

THE testis, if you will excuse the pun, has become a rather 'sexy' tissue to study, and for good reason. If the recently publicized reports of dramatically falling sperm counts in a range of animals prove correct, there is a clear need to improve our understanding of how spermatogenesis is regulated. Moreover, the way in which germ cells develop provides a whole set of unique questions relating to control of

complex developmental pathway activated by the testis-determining gene.

The primary regulators of spermatogenesis are hormonal in nature, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) acting on the Leydig and Sertoli cells respectively. Appropriately, then, two chapters are devoted to the way in which these hormones function, with a further chapter entitled "Paracrine Mechanisms in Testicular Control" outlining what is known about the way in which Leydig and Sertoli cells communicate with each other and with the germ cells. The ideas and experiments described here are pivotal to our understanding of spermatogenesis. The hormonal signals received by the testis are relayed by second messengers and growth factors to the developing germ cells. The line of communication is not unidirectional, however, and good evidence supports the idea that the germ cells also play a role in controlling Sertoli cell function. Like most of the other chapters in the book these chapters on hormonal influences are extensively referenced and punctuated by clear, well annotated diagrams and photographs.

Unsurprisingly the pathway of cellular differentiation present in the seminiferous tubules has tempted many to ask whether their favourite gene is transcribed during spermatogenesis. Two chapters in this book address this rather complex topic. Oncogenes, homeobox genes and others too numerous to mention have been used to probe northern blots of germ-cell RNA with, in most cases, the detection of transcripts. In the vast majority of cases these observations have done little to increase our understanding of how spermatogenesis is regulated but can be seen as the first step towards this goal. Both chapters dealing with gene expression during spermatogenesis act as an informative catalogue of what has and has not been studied in this context. Analysis of this issue is continually dogged by the possibility of functionally meaningless gene expression occurring as the result of chromosome condensation in preparation for DNA packaging.

The final complement of chapters is provided by discussions of growth factors in testicular function, signalling in spermatozoa, iron transport (highlighting the problems and benefits imposed by the blood-testis barrier) and vascular controls in testicular physiology. Those of us with an active research interest in the testis will find *Molecular Biology of the Male Reproductive System* a most accessible update on our current understanding of the subject. It would, however, be a shame if a book of this quality did not gain a wider, non-specialist readership. □

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Structural perspectives

David M. J. Lilley

Understanding DNA: The Molecule and How it Works. By C.R. Calladine & H. R. Drew. Academic Press: 1992. Pp. 220. £15, \$32.50.

DNA-Protein Interactions. By Andrew Travers. Chapman & Hall: 1993. Pp. 180. £14.95, \$44.

DNA Topology. By A. D. Bates and A. Maxwell. IRL Press: 1993. Pp. 114. £8.95, \$15.95.

Unraveling DNA. By Maxim D. Frank-Kamenetskii. VCH: 1993. Pp. 205. £20, DM40.

THERE has been an immense amount of activity in the areas of DNA structure and DNA-protein interactions since the beginning of the 1980s. This has largely come about because of the availability of high-resolution structures of DNA, proteins and their complexes using crystallography and nuclear magnetic resonance. The pace of ideas in the field has been hard to keep up with, and students' reading lists have grown ever longer. It has therefore been clear for some time that these subjects are fertile matter for textbooks, and the lack of such books has been a problem for undergraduate teaching. Now, like the proverbial London buses, four arrive at once. Yet despite a significant overlap in subject matter, it would be hard to imagine four more different books.

Of the four, *Understanding DNA* by Chris Calladine and Horace Drew is undoubtedly the most stylish. This is a book that the authors were clearly bursting to write, and it shows because their characters are indelibly imprinted on it. Their theme is that most of the structural properties of DNA can be derived from a consideration of the chemistry and interactions of the base pairs. From these assumptions, and some simple geometry, Calladine and Drew demonstrate that nucleic acids naturally adopt roughly 11-fold helices. Going a little deeper into the analysis they introduce propeller twist, and show how this results in certain classes of structure for different sequences. This then leads to a consideration of DNA curvature by oligoadenine tracts. The book is beautifully crafted, with a logical step-by-step approach to the subject. The view of DNA essentially from the point of view of the bases alone will not meet with universal approval, but it will provide valuable stimulation to thinking in the area. The result is a book from which the advanced undergraduate will benefit, and which will also generate a refreshing perspective for experts. The tone is a touch patronizing in places for some tastes, but

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Woody Allen as a sperm, from the film *Everything You Always Wanted to Know about Sex but Were Afraid to Ask*.

gene expression: how is cellular differentiation controlled so precisely both across and along the seminiferous tubule, what is the significance of the germ-cell syncytia and how do the final stages of sperm formation occur in the absence of transcription?

This book deals almost exclusively with mammals which, in view of the title, may cause some indignation among, for example, *Drosophila* biologists. That small reservation apart, the chapters have been well chosen and cover most of the important aspects of testis development and function. Appropriately, the first two chapters deal with events in the genital ridges leading to the formation of the testis, giving some of the latest ideas about how the SRY gene may be functioning. The juxtaposition of these chapters leaves the reader in no doubt about the highly

United Artists (Courtesy Kobal)

there is no denying that *Understanding DNA* is an accurate title.

Our structural understanding of DNA-protein interactions has increased immeasurably in the past decade, and a good textbook in this area has been needed for some time. The only problem is that the very pace of events in the field makes reviewing it rather like trying to photograph an express train head-on. *DNA-Protein Interactions* by Andrew Travers makes a brave attempt to cover the subject matter up to the pre-helix-loop-helix stage. Unfortunately the result somehow fails to capture the excitement of the area. The format does not help. Despite the very structural basis of the subject, there is no use of colour; all the figures are line diagrams, and some even have significant errors (such as the helix-that-isn't in Figure 1-11). But the book does bring together areas in a juxtaposition unique to its author, notably sequence phasing in the nucleosome, and the later sections discussing mechanisms of transcriptional regulation. These subjects are explained with the authority one would expect from Travers.

Although both of the above texts cover the supercoiling of DNA to some extent, another desperate need for those of us who teach DNA structure is a textbook covering the basic concepts in DNA topology. This is a subject that runs from the mathematical background through the physical chemistry of DNA, the enzymology and genetics of topoisomerases and even their importance in medicine. This is not an easy subject to present for under-

graduates, yet in *DNA Topology* I feel that Andy Bates and Tony Maxwell have succeeded brilliantly. They provide an extremely clear introduction to the required topological concepts, while not flinching from the rather more intellectually demanding area of surface linking, which is tackled with real competence. This small monograph is pitched at the right level, and pockets, for undergraduates, while I have noticed that it is already appearing on the desks of many graduate students and postdoctorals working in the area.

Unraveling DNA is a very different book from the other three. Maxim Frank-Kamenetskii weaves a curious blend of history, biographical details and science, to cover the development of molecular biology from the influence of the physicists earlier in the century, through the central dogma and bread-and-butter molecular biology, to discussion of social issues raised by genetic engineering. Along the way we have some diversions into the more structural areas of interest to the author, such as DNA structure and topology, and a refutation of the side-by-side model of DNA (does anyone take this seriously any more?). It is hard to know exactly for whom this book is intended. I couldn't seriously recommend it to university students, but there is no denying that it makes entertaining reading. I actually found it quite hard to put down. □

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Psychological diversity

Peter Bryant

Multiple Intelligences: The Theory in Practice. By Howard Gardner. *Basic Books*: 1993. Pp. 304. \$30 (hbk); \$15 (pbk).

TEN years ago Howard Gardner, who is a well-known Harvard psychologist, wrote a book that gave a revolutionary account of intelligence. The book was in part an attack on traditional approaches to intelligence and in part an attempt to set up an entirely new approach. Gardner's main worry about the traditional view of intelligence was the insistence of many psychologists on the existence and the pervasive importance of g, an overarching general factor which plays a role in every activity that could be called intelligent.

The evidence for g is the persistent correlation that has always been found between the highly diverse tasks that make up the typical IQ test. For example, if one asks the same group of children to define the meaning of some reasonably

common words and also to form some patterns with coloured bricks, one will definitely find a very strong relationship between their successes in these two apparently heterogeneous tasks. The two tasks measure intelligence in the sense that both are related to children's academic performance at school, and the fact is that all tasks that fall into this category correlate quite strongly with each other. Many, though not all, psychologists concluded from this that the reason for the persistent correlation between all successful intelligence tasks is that children's performance in each of them is determined by the same underlying general factor of intelligence which they called g.

There were those who disagreed with this idea and argued for the existence of a set of specific and independent intellectual abilities. These dissidents tried their hardest to get rid of the correlation by devising new measures that represented these hypothetical abilities more closely,

but they never succeeded. Their new tests correlated as stubbornly and as strongly as the old ones did; g, it seemed, was here to stay.

In his 1983 book (*Frames of Mind*, Heinemann), Gardner pursued the same dissident line but mounted an altogether different kind of attack on g. To justify his idea of "multiple intelligences", he pointed to the existence of some people with remarkable talents and also to the highly specific effects of brain damage. The fact that some people develop one particular skill (such as musical, literary, artistic or mathematical) to an astonishing degree and yet remain quite ordinary in other intellectual areas is evidence, Gardner argued, for intellectual abilities that are quite independent from one another. He advanced much the same argument for the highly specific effects of various forms of brain damage: the discovery that one aspect of a person's intellectual life is completely knocked out by damage to the brain while the rest stays intact suggests, according to Gardner, the existence of an independent and specific intellectual skill.

In place of g, Gardner advanced the notion of a set of seven quite independent intelligences: these were in nature musical, bodily kinaesthetic, logical-mathematical, linguistic, spatial, interper-

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How intelligent are IQ tests?

sonal and intrapersonal. But he did not attack the notion of g head-on by trying to find measures of the purported independent abilities which, therefore, do not correlate with one another.

In his new book, aptly called *Multiple Intelligences*, Gardner returns to the same hypothesis, and in the intervening years, it seems, he has done a great deal to gather data for it. The book starts with two chapters that reiterate the idea of seven independent forms of intelligence. Then Gardner goes on to attack g. He argues that it exists, but only because current intelligence tests are too limited. They concentrate, according to Gardner, on linguistic and logical intelligence and do

not touch other aspects of intellectual life. They correlate with each other, therefore, because they all measure the same limited thing.

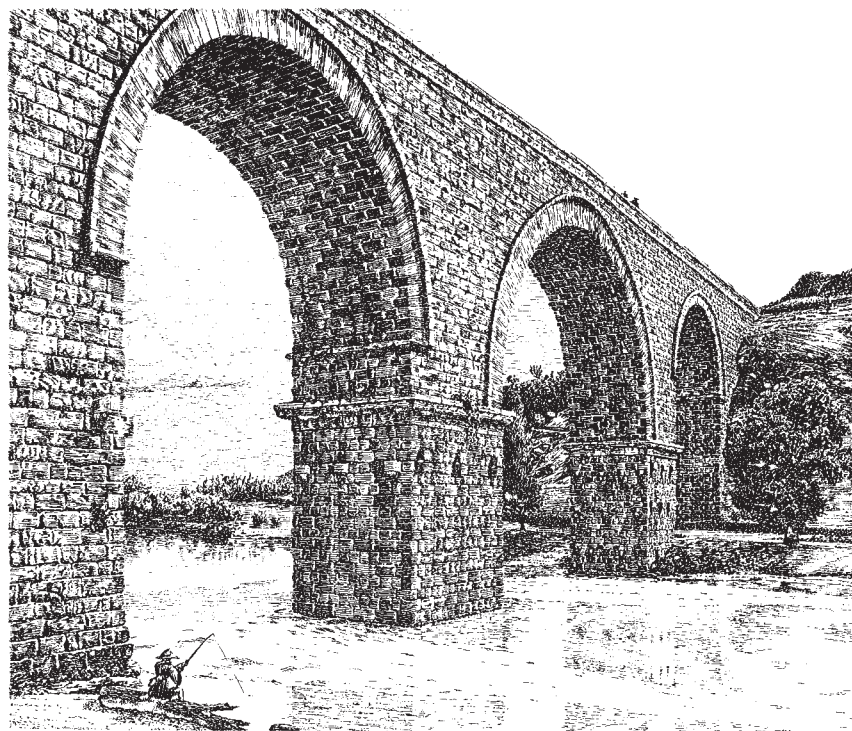
He then gives an account of some more wide-ranging tests that he has developed to measure the seven intelligences. He reports that preliminary data suggest that these measures are indeed independent in that for the most part they do not correlate with each other. But the data, only sketchily described in this book, are indeed very preliminary. They come from no more than a handful of children, far too few for proper research on any psychological measuring instrument. As Gardner himself says: "Because of the small sample that received the Spectrum battery, the study should be regarded as generating hypotheses rather than as conclusive in any sense" (p.105).

Another problem is that we do not yet know whether these tests are genuinely measures of intelligence, because Gardner's research team has not so far shown how well the tests predict children's achievements outside the testing situation. "We do not know yet whether a Spectrum assessment can predict scholastic success with the reliability of standard forms of assessment" (p.106).

The other main theme of this new book is the relevance of the theory of multiple intelligences to education. The claim that Gardner makes is a simple one: "To the extent that there are electives, it is pertinent for students to know their own proclivities" (p.73). If children are to specialize at school, he argues, they need the help of professionals: they need an "assessment specialist" who will talk to a "curriculum broker" and together they will decide the most appropriate set of lessons for each child. But the strength of Gardner's arguments about the management of specialization in schools depends in the end on the success or failure of his ambitious and still unproved theory about multiple intelligences. We need many more data before we can accept these new ideas.

In the meantime some of the observations in this new book are particularly acute and make it worth reading. I liked Gardner's account of children's conceptions of different school subjects. "In science," one child told him, "you learn about nature: in English they are teaching about how to talk properly, like 'I learned about frogs today: ain't that nice?' — that's not good English, but it's okay to say in science" (p.126). Here at any rate is a youngster who, like Howard Gardner, believes in the independence of different intellectual activities. □

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RECONSTRUCTION of the Ponte d'Augusto on the Via Flaminia at Narni, Italy. From *Roman Bridges* by Colin O'Connor. Cambridge University Press, £65, \$100.

C. O'Connor

Fossils through time

Bob Savage

The Fossil Record 2. Edited by M. J. Benton. *Chapman & Hall*: 1993. Pp. 845. £95.00. \$149.95.

WHEN Linnaeus published *Systema Naturae* (1758), wherein all known species of plant, animal and mineral are given binomial Latin names, he included Petrifications. He named some 40 fossils, most of them molluscs; in contrast, he named more than 12,000 living species. Today, about 100,000 palaeospecies are known over a span exceeding three billion years, yet many gaps remain. So many data have accumulated that in 1965 a committee of the Geological Society of London decided to review the fossil record, with particular reference to the ranges of taxa. It is that work which is now superseded by *The Fossil Record 2*.

The taxonomic unit chosen to record fossils by first and last appearances is the family. Its status may not equate across all phyla, but it is a sound choice because genera are too numerous and orders are too long-ranging. Over 7,000 families are listed, more than double the number in the first edition. This is due partly to new research, but also to fine tuning (many orders were undivided in the first edition). The ranges are mostly resolved to strati-

graphic stages (stage durations average 5 million years). Only marine stages are listed and no equivalents are given for terrestrial taxa. Each chapter has a bibliography, and the index lists taxa only under vernacular names, which do not appear elsewhere. Charts plot the time ranges of each family and, wisely, no attempt is made to interpret phylogenetic relationships. Fossils in all phyla are recorded, together with Monera, Fungi, 'Algae', Problematica and Miscellanea.

The editor and authors of this massive database are to be congratulated on a very substantial achievement. Its strong points are the comprehensive coverage, the up-to-date accurately recorded data and consistency in use of family units and stratigraphic stages. The first edition concluded with histograms of turnover rates. Modern computer programs can generate highly sophisticated analyses, and it is for such ends that the immense labour of producing the tome is convincingly justified. Research on rates of evolution and extinction can now be fuelled by this great compilation. The authors' labours will not have been in vain. □

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Direct observation of structure in the cosmic microwave background

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Temperature fluctuations in the cosmic microwave background are a key prediction of cosmological models of structure formation in the early Universe. Ground-based multi-frequency radio observations of the microwave background reveal well defined features that are not of atmospheric or Galactic origin; instead, they delineate the primordial density perturbations from which the structure in the present Universe originated. The amplitude of these perturbations confirms the earlier statistical detection by the COBE satellite.

THE detection and mapping of fluctuations in the 2.7-K cosmic microwave background (CMB) is motivated by the desire to understand the origin of the structures observed in the present-day Universe. The seeds of such structures imprint fluctuations onto the CMB some 300,000 years after the initial singularity. At this epoch, the Universe has cooled sufficiently for free electrons and nuclei to combine into neutral atoms, allowing the photons to decouple from the matter distribution. Effectively, we obtain a snapshot of the Universe at this time, providing us with a unique test of cosmological models.

In practice, observations of the CMB are limited by local emission from both the Earth's atmosphere and the Galaxy, thus necessitating carefully designed experiments in order to discriminate against these unwanted foregrounds. Here we report on the analysis of CMB observations made using the ground-based 'Tenerife experiments' at the Observatorio del Teide, and it will be seen that not only do our new data detect CMB fluctuations, in agreement with the statistical detection by the COBE satellite, but for the first time allow the identification of real, individual, primordial features in the CMB.

The design and construction of the Tenerife instruments¹ has been undertaken in such a way as to provide maximum sensitivity to astronomical signals, whilst at the same time reducing atmospheric noise and spillover effects to an acceptable minimum. The experiments, which are operated by the University of Manchester in collaboration with the IAC Tenerife, consist of three fully independent, dual-beam (5.5° full-width half-maximum, FWHM), double-switching, drift-scan radiometers observing at frequencies of 10.4, 14.9 and 33.0 GHz. The physical size of the instruments is scaled so as to produce the same triple beam pattern² on the sky at each observing frequency. This allows direct comparison of the multi-frequency observations and thereby provides the discriminatory power essential to distinguish CMB fluctuations with a blackbody spectrum from possible foreground contaminants. The addition of a third frequency at 33 GHz, where the Galactic emission is substantially lower, offers a major advance over our previous experiments^{2,3}.

Our strategy is to make repeated high-sensitivity drift-scan observations over selected areas of low Galactic emission, and to stack these together to provide improved sensitivity to astronomical signals. This contrasts with large-scale survey instruments such as COBE, which map the whole sky but with a consequent reduction in sensitivity to sky features. The basis of the COBE anisotropy detection⁴ rested not on the observation of individual CMB features, but rather on the fact that the statistical treatment of the COBE data indicated signals greater

than expected from instrumental noise. By contrast, the new Tenerife data provides a direct detection of hot and cold spots in the CMB. It will be shown that the r.m.s. (root mean square) signal deduced from the COBE data is consistent with our detection, and as the two experiments are made in different regimes of Galactic contamination, the agreement gives further confidence in the results.

CMB observations on a given angular scale sample a region of the power spectrum of fluctuations, whose form is predicted by any viable cosmological model. The power spectrum of the seed perturbations is conventionally taken to have a power-law form $P(k) \propto k^n$ (k = co-moving wavenumber). If, as is commonly hypothesized^{5,7}, the Universe went through a phase transition very early in its history ($t < 10^{-34}$ s), then the vacuum energy released by this would cause the Universe to expand exponentially, inflating quantum fluctuations to produce these seed structures. In such inflationary scenarios, the fluctuations are expected to have equal power on all scales, giving a so-called Harrison-Zel'dovich spectrum, for which $n = 1$. In contrast to standard Big Bang scenarios, for which there is no causal mechanism for the production of fluctuations on scales greater than the horizon size at recombination ($\sim 2^\circ$), inflationary models thus predict coherent seed perturbations to exist even on the large angular scales of the Tenerife CMB observations. The results presented here offer the first direct observations of CMB features corresponding to seed structures in the early Universe. The amplitude inferred for the seeds is as expected if the mechanism of structure formation is by way of gravitational instability. A combined treatment of the Tenerife and COBE detections provides a limit on the spectral index of fluctuations of $n \geq 0.9$, and is in accordance with inflationary models for the origin of structure in the Universe.

Observations and data reduction

In this section we describe the basic observing technique and the reduction of the data to a form suitable for further analysis. The sky area for observation was selected under the constraints imposed by local Galactic emission and the drift-scanning method, and covers a 360° strip in right ascension (RA) at a fixed declination of +40°. Each instrument simultaneously records data through two independent receiving channels, denoted channel A and channel B, so that for a single passage of the instrument beams across the sky, two scans result. Daily calibration was achieved from the magnitude of the Galactic plane crossing, while on-line noise injection diodes ensured a stable calibration throughout an observing cycle. Absolute cali-

TABLE 1 Statistical analysis of the 10, 15 and 33 GHz data

ν (GHz)	σ_s (μ K)	$\sigma_{(A+B)/2}$ (μ K)	$\sigma_{(A-B)/2}$ (μ K)	$\chi^2_{(A+B)/2}$	$P(\chi^2_{(A+B)/2})$	$\chi^2_{(A-B)/2}$	$P(\chi^2_{(A-B)/2})$
10	36 ± 116	190 ± 21	186 ± 21	19.9	0.22	10.7	0.86
15	41 ± 24	94 ± 10	85 ± 10	29.6	0.02	11.9	0.75
33	49 ± 10	66 ± 6	44 ± 6	45.7	1×10^{-4}	22.0	0.14
15+33	42 ± 9	58 ± 5	40 ± 5	55.9	3×10^{-6}	18.0	0.32

Statistical analysis of the 10, 15 and 33 GHz data (15+33 is the weighted average of 15 and 33 GHz) covering RA 161° – 230° , by splitting into independent subsets A and B (see Fig. 1). Columns 1–4; columns 3 and 4 contain the r.m.s. of the $(A+B)/2$ and $(A-B)/2$ data respectively. The 4° (RA) difference in the variance of the $(A+B)/2$ and the $(A-B)/2$ data is due to the presence of an astronomical signal characterized by the r.m.s. displayed in column 2, $\sigma_s^2 = \sigma_{(A+B)/2}^2 - \sigma_{(A-B)/2}^2$. These values are for data binned at 1° intervals in RA and σ_s corresponds to an estimate of the astronomical structure as smoothed by the Tenerife triple beam pattern. It is evident that there is structure present at a similar amplitude in both the 15 and 33 GHz data. Columns 5–8; The χ^2 values (columns 5 and 7) for the 4° binned data as displayed in Fig. 1 confirm that the $(A-B)/2$ data do indeed correspond to a random noise distribution, whereas the $(A+B)/2$ data display definitive evidence of an astronomical signal. $P(\chi^2)$ (columns 6 and 8) is the probability of obtaining this value of χ^2 (for 16 degrees of freedom) under the null hypothesis of no signal being present in the data.

bration was implemented by a series of observations of the Sun, the Moon and microwave absorber. The contribution of the Sun and Moon was reduced to an insignificant level by flagging and removing data taken at $\lesssim 50^\circ$ from the beam axes. Additional flagging removed data which deviated from the mean by ≥ 3 standard deviations for any given scan, and data were also rejected when poor observing conditions caused excessive drifts of the mean scan level. Before stacking the calibrated and edited data scans, it is necessary to remove the non-astronomical baseline drifts caused by humidity gradients in the atmosphere (A.N.L. *et al.*, manuscript in preparation). Subtraction of these baselines from the data then allows one to stack together N daily scans free of baseline drifts to produce a final scan at each frequency with a $\sqrt{N}$ improvement in the noise. We select the region of minimum Galactic and radio source emission for detailed analysis, thus defining the region RA 161° – 230° at

declination $+40^\circ$ (corresponding to Galactic latitude $b > 47^\circ$).

As detailed in Watson *et al.*², the contribution of the discrete radio sources was simulated using the Kühr catalogue⁸ and the region considered is found to be essentially uncontaminated by such point sources, to a maximum level of 8μ K (r.m.s.) at 15 GHz and 3μ K at 33 GHz; Fig. 1a, c and e shows the scans after correction for this radio source component. Common structure is clearly present in the scans at each frequency and represents a combination of intrinsic CMB fluctuations and possible diffuse Galactic emission. The improvement in the data over and against those reported by Watson *et al.*² is clear, with the previous best standard error per beam of 35μ K at 15 GHz being reduced to 29μ K. This in turn has been eclipsed by the latest 33-GHz instrument which now achieves a noise level of 20μ K per beam area. Thus it is seen that observations about the minimum in Galactic emission produce high sensitivity scans

containing structure that is not attributable to discrete radio sources.

Statistical analysis

A quantitative determination of the significance of the structure visible in the data scans will now be made by splitting the data into subsets to be analysed independently, and also by applying a more sophisticated likelihood analysis. At both 10 and 33 GHz the full data set was split into equal halves, one from channel A and the other from channel B. At 15 GHz, the usefulness of the split by channel was limited by the failure of the channel B receiver for $\sim 30\%$ of the observing cycle, so the data were split into two epochs with equal numbers of observations in each. As may be seen in Table 1, the results of the data splitting confirm that we are seeing real structure on the sky which may be Galactic or cosmological in origin. A terrestrial origin is considered highly unlikely given the independence of the experiments and the precautions taken in their design and operation¹. The possibility of the structure being residual atmospheric signals is implausible because the spectrum of the signals is inconsistent with atmospheric emission, and also the observed structure is seen to repeat spatially between the independent experiments operating over different time periods. We now expand on these results with a full likelihood analysis of the data scans after removal of discrete radio sources.

Likelihood analysis. In our experiments

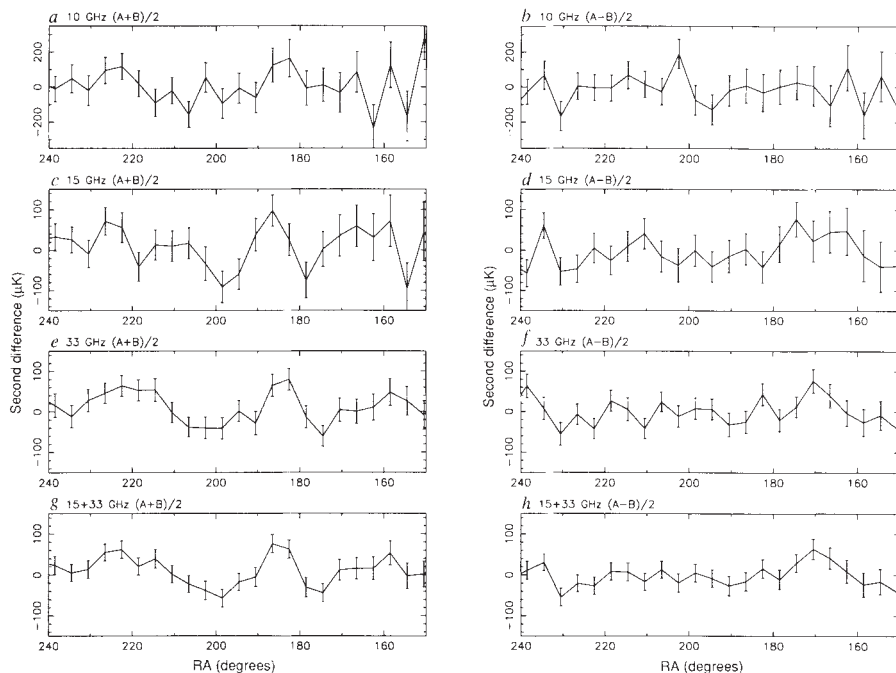


FIG. 1 The stacked data scans at each frequency. The observed second difference in sky temperature is plotted against the position in RA. The $(A+B)/2$ data (a, c and e at 10, 15 and 33 GHz respectively) contain the signal plus instrument noise: the $(A-B)/2$ data (b, d and f) represent the instrument noise. The $(A+B)/2$ scans result from the linear combination of the scans from the independent analysis of equal splits A and B of the data. At 15 GHz split A constitutes the summer observing season (June, July, August) and the split is a useful consistency check on the absence of residual seasonal effects. The $(A+B)/2$ data have been corrected for emission from discrete radio sources. The one standard deviation error bars on a single 4° bin in RA are calculated from the variance over the daily scans. g and h, the weighted average of the 15 and 33 GHz data.

TABLE 2 Results of likelihood analysis

ν (GHz)	Detected $\sqrt{C_0}$ (μK)	Detected $Q_{\text{rms-ps}}$ (μK)
10	29^{+20}_{-30}	15^{+9}_{-12}
15	48^{+16}_{-16}	23^{+10}_{-8}
33	60^{+16}_{-16}	29^{+8}_{-7}
15 + 33	54^{+14}_{-10}	26^{+6}_{-6}

Results of the likelihood analysis at each frequency (15 + 33 is the weighted average of the 15 and 33 GHz data). Column 2 contains the detected r.m.s. signal level for intrinsic fluctuations described by a gaussian model of the auto-correlation function with coherence angle $\theta_c = 4^\circ$ (for which the Tenerife experiments have optimum sensitivity to astronomical fluctuations). For a Harrison-Zel'dovich spectrum of fluctuations the detection is characterized by the quadrupole moment, as tabulated in column 3. The quoted errors are at 68% confidence and have been computed by bayesian integration (uniform prior) under the likelihood curve to the 68% area points.

the data points are correlated because the intrinsic astronomical fluctuations are correlated and because correlations are introduced by the telescope sampling function. Using the likelihood approach^{2,9}, the method effectively deconvolves the switching correlations and has the benefit of allowing one to directly test given astronomical models. Theoretical models of structure formation predict the power spectrum of the astronomical fluctuations, which then defines the intrinsic angular auto-correlation function,

$$C_{\text{intr}}(\theta) = \langle \delta T(\mathbf{q}_1) \delta T(\mathbf{q}_2) \rangle$$

where $\delta T(\mathbf{q})$ is the fluctuation amplitude in direction $\mathbf{q}$, and $\mathbf{q}_1 \cdot \mathbf{q}_2 = \cos(\theta)$. This function fully specifies all the statistical information on the CMB fluctuations for models that may be described by a random gaussian field. Other models such as cosmic strings^{10,11}, are not considered here. Two choices for the form of the intrinsic sky auto-correlation function $C_{\text{intr}}(\theta)$ have become common. First, a gaussian-shaped function, in which only a height and width have to be specified. This is useful for comparing with the results of other experiments on intermediate^{12,13} and small¹⁴ angular scales, although its extension to the large angular scales of COBE is not necessarily physically realistic¹⁵. If we take $C_{\text{intr}}(\theta)$ to be a gaussian function with amplitude $\sqrt{C_0}$ and coherence angle θ_c , then after convolution with the Tenerife beam (dispersion σ) we obtain³ an observed auto-correlation function:

$$C_{\text{obs}}(\theta, \sigma) = \frac{C_0 \theta_c^2}{2\sigma^2 + \theta_c^2} \exp\left(\frac{-\theta^2}{2(2\sigma^2 + \theta_c^2)}\right)$$

Second, a power-law form for the power spectrum of the fluctuations, $P(k) \propto k''$. By expressing $C_{\text{intr}}(\theta)$ in terms of a spherical harmonic expansion

$$C_{\text{intr}}(\theta) = \frac{1}{4\pi} \sum_{l \geq 2} a_l^2 (2l+1) P_l(\cos \theta)$$

and convolving with a gaussian beam of dispersion σ one obtains¹⁶,

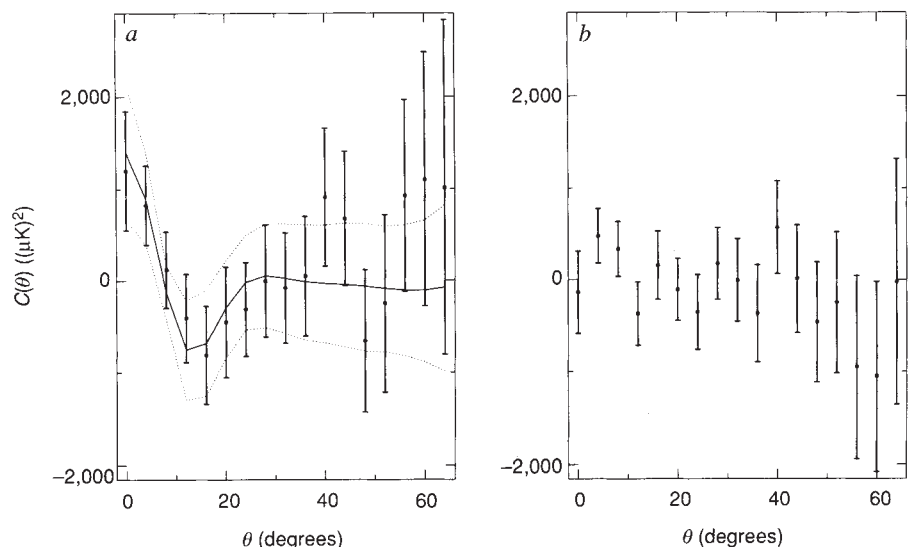
$$C_{\text{obs}}(\theta, \sigma) = \sum_{l \geq 2} (2l+1) P_l(\cos \theta) \exp(-[(l + \frac{1}{2})\sigma]^2) \times \left(\frac{\Gamma(l + \frac{(n-1)}{2}) \Gamma(\frac{(9-n)}{2})}{\Gamma(l + \frac{(5-n)}{2}) \Gamma(\frac{(3+n)}{2})} \right) \frac{Q_{\text{rms-ps}}^2}{5} \quad (1)$$

$Q_{\text{rms-ps}}$ is conventionally taken as the normalizing factor for this form of power spectrum, and is related to the quadrupole amplitude, a_2 , through $Q_{\text{rms-ps}}^2 = 5a_2^2/4\pi$.

Given a model for the astronomical auto-correlation function it is then possible to use the likelihood function to calculate the most probable model parameters, with the appropriate confidence levels. We evaluate the likelihood function for the relevant covariance matrix, to obtain the likelihood of obtaining the observed set of data $\Delta T = (\Delta T_1, \Delta T_2, \dots, \Delta T_n)$ given some model intrinsic auto-correlation function $C_{\text{intr}}(\theta)$. The total covariance matrix contains all of the information on correlations due to the switching and intrinsic astronomical structure, along with the contribution of the random errors on the data². The procedure is to vary the parameters of the model to obtain different values of $C_{\text{intr}}(\theta)$ and then to calculate the likelihood of observing the data set ΔT . The most probable model parameters can thus be determined and a detection of fluctuations will manifest itself as a peak in likelihood away from zero when varying the amplitude parameter of the fluctuations.

The likelihood test was applied to the 10 GHz, 15 GHz and 33 GHz data, in the range RA 161° – 230° , after correction for discrete radio source emission. For the case of a gaussian model auto-correlation function with $\theta_c = 4^\circ$ (the angle at which the Tenerife experiments achieve their highest sensitivity), the results

FIG. 2 The cross-correlation function $C(\theta)$ for the 15 GHz and 33 GHz (A+B)/2 and (A-B)/2 data. a, (A+B)/2 (15 GHz) $\times$ (A+B)/2 (33 GHz). b, (A-B)/2 (15 GHz) $\times$ (A-B)/2 (33 GHz). $C(\theta)$ was calculated using $(\sum_{i,j} \Delta T_i \Delta T_j w_i w_j) / (\sum_{i,j} w_i w_j)$ for all temperatures $\Delta T_i, \Delta T_j$ subtending a mutual angle θ in RA at declination $+40^\circ$, with weights $w_i = 1/\sigma_i^2$. The error bars represent the 68% confidence limits on the data points. The continuous lines are the auto-correlation function for a Harrison-Zel'dovich spectrum, as convolved with the Tenerife beam and normalized to $Q_{\text{rms-ps}} = 26 \mu\text{K}$. The dotted curves represent the 68% error bounds due to cosmic variance and limited sky coverage, as computed from Monte Carlo simulations (see text and Fig. 3).



are given in column 2 of Table 2. Taking the special case of a Harrison-Zel'dovich power spectrum, for which $n=1$ (that is, $P(k) \propto k$), one obtains the results in column 3. For each independent data set, at each frequency, structure is detected for both of the astronomical models under consideration, as one would expect from the elementary excess noise analysis of Table 1. The most significant detections are attained in the most sensitive 15 and 33 GHz data and these occur at 3–4 standard deviation confidence. The amplitudes of the detections are the same at each frequency, as expected if the structure is of common origin, and has a blackbody spectrum.

Cross-correlation analysis. Structure of common origin, such as CMB fluctuations, will correlate between the independent scans at the different frequencies. The degree and significance of the correlation between the different data sets in Fig. 1 was investigated with reference to both the simple Pearson r -test and the full cross-correlation function. The r -test was applied to all combinations of 'data $\times$ data' (that is, $(A+B)/2 \times (A+B)/2$) and also to 'noise $\times$ noise' (that is, $(A-B)/2 \times (A-B)/2$). In each case, the $(A+B)/2 \times (A+B)/2$ data exhibit significant positive correlations over the RA range 161° – 230° . For example, for $33_{(A+B)/2} \times 15_{(A+B)/2}$ one obtains $r = +0.49 \pm 0.17$, with the null hypothesis being rejected at $\geq 95\%$ confidence. Conversely all combinations of $(A-B)/2$ and $(A-B)/2$ demonstrate poor correlation as expected for noise alone. One can conclude that the structure detected at each frequency has common spatial characteristics and is thus of common origin.

Furthermore, the cross-correlation function for the 15 GHz and 33 GHz $(A+B)/2$ data, as plotted in Fig. 2, is inconsistent with that for noise, at 95% confidence (over the angular range 0 – 20° for which the experiments are sensitive to astronomical structure), whereas the $(A-B)/2$ data is consistent with a random noise distribution. Again this confirms that at least a fraction of the statistical detections at these frequencies originate from structure that is common to the individual scans. Additionally, the value of the cross-correlation function at zero lag agrees with that for the auto-correlation function of both the 15 GHz and 33 GHz data separately, thereby implying that the component of correlated structure at 15 and 33 GHz has the same amplitude.

Origin of the structure

Having verified that the structure detected at each frequency is of common origin, the multi-frequency nature of the data set allows one to discriminate between structure with a blackbody spectrum (characteristic of CMB anisotropy) and that of Galactic origin. Crucial in this regard is the good spectral discrimination afforded by observations at 10 GHz, 15 GHz and 33 GHz. Dust emission being negligible at $\nu \lesssim 33$ GHz, the Galactic contaminants^{17,18} that may be significant are synchrotron and free-free radiation, resulting from the acceleration of relativistic electrons by magnetic fields and by interactions of thermal electrons with ions respectively. In both instances, the spectrum obeys

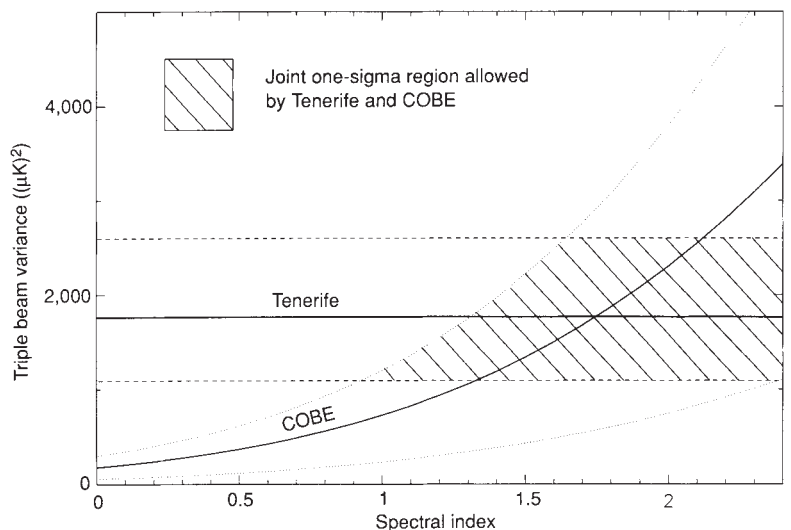
$$\frac{T(\nu)}{T(\nu')} = \left(\frac{\nu}{\nu'}\right)^{-\beta}$$

where β , the temperature spectral index, is ~ 2.1 for free-free, and ~ 2.8 for synchrotron radiation¹⁹.

Previous Tenerife results² have relied upon the extrapolation of low-frequency survey information^{20,21} over a decade in frequency in order to estimate the Galactic component of the signal at 10 and 15 GHz. This has serious problems due both to scanning errors and baseline inaccuracies in the low frequency surveys, which essentially render such predictions useless (R.D.D. and R.A.W., manuscript in preparation). The current high-quality 33 GHz observations now allow a spectral analysis using the Tenerife data alone. If we consider the worst case situation of all of the signal at 10 GHz being due to free-free emission, then taking the detection 29^{+20}_{-30} μK (for a gaussian auto-correlation function) as an illustration of the Galactic emission, implies an r.m.s. free-free component of less than 4 μK at 33 GHz. Clearly this is at variance with the detected signal of 60^{+16}_{-16} μK , and implies that less than 7% of the signal at 33 GHz is of Galactic origin.

Chi-squared fitting as applied to the three different spectral candidates supports the conclusion that the principal signal has a blackbody spectrum. Taking the data points from column 2 of Table 2, the χ^2 for each of the blackbody, synchrotron ($\beta = 2.8$) and free-free ($\beta = 2.1$) fits are 1.6, 17.7 and 15.5, with probabilities 0.45, 1×10^{-4} and 4×10^{-4} respectively. The best fit is

FIG. 3 Constraints on the spectral index n of fluctuations from a comparison of the Tenerife detection level with that of the COBE results. The variance in a Tenerife triple beam (σ^2) necessary for agreement with the COBE results, is plotted as the solid curve, bounded by the one standard deviation confidence limits (dotted curves). Comparison of this predicted triple beam variance with the observed value of $\sigma_s = 42 \pm 9$ μK (horizontal lines) defines the shaded region of n space allowed by the combined observations. On the Tenerife and COBE angular scales the perturbations are directly imprinted on the CMB by means of the Sachs-Wolfe effect²⁷ and their spectrum reflects that of the initial seed fluctuations. Thus one can use the power-law representation for the power spectrum to simply scale the COBE detection level to the Tenerife angular scales (see, for example, ref. 28). Here we normalize to the published COBE detection of $Q_{\text{rms-ps}} = 16.3 \pm 4.6$ μK at $n = 1.15$, and scale as a function of n as described in Fig. 3 of ref. 15. Using equation (1) and convolving with the $-\frac{1}{2}, +1, -\frac{1}{2}$ switching pattern (θ_b being the switching angle), one obtains the expected variance in a triple beam, for any given values of n , $\sigma_s^2 = \frac{1}{3}C_{\text{obs}}(0) - 2C_{\text{obs}}(\theta_b) + \frac{1}{3}C_{\text{obs}}(2\theta_b)$. In order to consider the uncertainty introduced by describing the Universe by a statistical model (cosmic variance), and the fact that the limited sky coverage of the Tenerife experiments may not be truly representative of the sky as a whole, we use Monte Carlo techniques to produce a large number (here 5,000) of realizations of skies



described by a power-law power spectrum as normalized to the COBE detection. By then replicating the Tenerife instrument sampling strategy for each realization one can predict the r.m.s. signal level and its confidence bounds for the above range of n . Taking a joint one standard deviation bound for both experiments the detections are seen to be consistent for $n \geq 0.9$ and favour $n = 1.7$.

to a blackbody signal of 48^{+11}_{-9} μK . A Galactic origin is ruled out even after consideration of the possible errors ($<10\%$) in the relative calibration between the different frequencies.

Taking CMB anisotropy as the most consistent and conservative explanation of the observations, then a weighted combination of the data scans from each frequency will provide the best information on CMB anisotropy. We exclude the 10 GHz data from this addition because of concern over residual Galactic emission at this lower frequency and assume for the present that all of the signal at 15 and 33 GHz is attributable to CMB (a detailed Galactic separation will be presented in a forthcoming paper). The weighted average of the 15 and 33 GHz data is depicted in Fig. 1g and agrees in form with the individual (A+B)/2 data scans. The excess-noise analysis and likelihood analysis of this 'best' scan, give $\sigma_s = 42 \pm 9$ μK (see table 1), $\sqrt{C_0} = 54^{+14}_{-10}$ μK and $Q_{\text{rms-ps}} = 26^{+6}_{-6}$ μK , constituting a 4–5 standard deviation detection of structure, independent of the particular analysis method. The standard error in a 5.5° beam area for this scan is 16.5 μK and it is clear that there are well-defined structures present in the data. In particular, the features over RA 170° – 210° delineate hot and cold spots (as convolved in the triple beam pattern) in the CMB that are a direct result of primordial seed structures.

Comparison with results from COBE

Comparable observations of CMB features by other experiments do not so far exist and we are therefore restricted to a basic comparison of the statistical amplitude of the fluctuations with the anisotropy detections published elsewhere. The most important of these are the COBE results and we leave comparison with other experiments to a forthcoming paper (S.H. *et al.* manuscript in preparation). The comparison provides an independent check on the COBE results, extending the angular scale of detection from $\theta \geq 10^\circ$ to $\theta \geq 4^\circ$ and improving the limits on n .

Figure 3 compares the Tenerife detection level in a triple beam of $\sigma_s = 42 \pm 9$ μK with that of the COBE observations, by assuming a power-law form for the power spectrum. The amplitude of the signal in the Tenerife data is seen to be consistent with that reported by the COBE group, under the constraint that $n \geq 0.9$. This lower limit may be compared with $n = 1.0 \pm 0.6$ obtained by Smoot *et al.*⁴ from fitting to the COBE data in isolation. The precise range of allowed n space is strongly dependent on the detailed nature of the comparison and although inflationary models are still allowed, our current observations are beginning to put pressure on such theories. The improvement from 0.4 to 0.9 in the lower limit on n is particularly significant in the context of inflationary theories of structure formation, because $n \approx 0.8$ is the point at which the gravitational wave, or 'tensor mode' contribution to CMB anisotropies is equal to the scalar or density perturbation contribution^{22,23}. For $n \geq 0.8$ the tensor contribution declines rapidly, meaning that the level of CMB fluctuations can be used directly to provide the level of normalization for use in theories of galaxy formation. The general level of CMB anisotropy, as now established by COBE, and this present work, is of great significance in constraining cosmological parameters, and distinguishing between competing models of galaxy formation. For example, the relatively low level of CMB anisotropy as compared to the density perturbations that are required at decoupling in order to produce the structures seen today, lends strong support to theories involving dark matter. This is both because the dark matter can cluster earlier than ordinary matter and because a larger value of the density parameter Ω is required in order to allow time for the observed structures to form. The value of n is also of great importance in the later stages of structure formation, because in conjunction with the nature of the dark matter (hot, cold or mixed; see, for example, ref. 24) it determines the degree of clustering as a function of linear scale, which can then be compared with the observations.

A Harrison–Zel'dovich spectrum ($n=1$) is allowed by our results, and implies a power spectrum normalization of $Q_{\text{rms-ps}} = 26 \pm 6$ μK for the Tenerife data. This value is compatible with that of $Q_{\text{rms-ps}} = 17 \pm 5$ μK , as reported by Smoot *et al.*⁴ for $n=1$. Repeating the Monte Carlo simulations as in Fig. 3, with $Q_{\text{rms-ps}} = 26$ μK , one can calculate the auto-correlation function and its error bounds for a Harrison–Zel'dovich spectrum. This is seen to be in accordance with the observed cross-correlation function as shown in Fig. 2. In a similar manner, one can make Monte Carlo simulations of a sky described by a Harrison–Zel'dovich spectrum with the COBE normalization and apply the likelihood analysis (assuming a gaussian sky auto-correlation function) to each realization. Three quantities which it is of interest to calculate from such simulations are: (1) the probability of obtaining *no* detection with our current experiments—if this is low it indicates that our experiments are indeed sensitive to structures at the COBE level; (2) the probability of a detection at the level we in fact find; and (3) the probability that an experiment with the same instrumental sensitivity and coverage as Watson *et al.*² would have found the reported upper limit, despite the presence of true astronomical fluctuations at the COBE amplitude. These probabilities are respectively 7%, 12% and 30%, indicating that our current experiments are indeed very sensitive, with a detection level that is higher than expected from the COBE results, although not significantly so, and that the Watson *et al.* upper limit is consistent.

The Tenerife data agree with the published COBE results, attaining a similar statistical sensitivity to structure, but over a much reduced sky area, with consequently improved sensitivity to given features. In the COBE first-year maps, the noise level per 10° FWHM beam area was ~ 45 μK with an r.m.s. signal of 30 μK . Consequently no single feature could be identified as being real rather than noise. In contrast, the Tenerife 15+33 scan achieves a noise per 5.5° beam of 16.5 μK for an r.m.s. signal of 42 μK and one can now clearly identify given CMB fluctuations. These CMB features do not correspond to structures visible today, the largest of which subtend $\leq 1^\circ$ in the CMB^{25,26}, but can be explained as the imprints of the large-scale seed perturbations expected from an inflationary power spectrum. Future improvements in the COBE data may allow a useful comparison of features with the current Tenerife results whilst improved data from either experiment will certainly narrow the range of allowed n space. The Tenerife experiments are continuing to observe and, by means of scans at adjoining declinations, have already extended the sky coverage from that reported here, by a factor of three at 10 and 15 GHz (C.M.G.C. *et al.*, manuscript in preparation). Future results should facilitate the mapping of fluctuations over a broader sky area and their comparison with theoretical sky maps should provide a new insight into structure formation in the early Universe. $\square$

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Structure of pentameric human serum amyloid P component

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The three-dimensional structure of pentameric human serum amyloid P component at high resolution, the first reported for a pentraxin, reveals that the tertiary fold is remarkably similar to that of the legume lectins. Carboxylate and phosphate compounds bind directly to two calcium ions; interactions with a carboxyethylidene ring are mediated by Asn 59 and Gln 148 ligands of the calcium ions. These X-ray results indicate the probable modes of binding of the biologically important ligands, DNA and amyloid fibrils.

HUMAN serum amyloid P component (SAP) is a decameric plasma glycoprotein composed of identical subunits non-covalently associated in two pentameric rings interacting face-to-face. SAP binds to all forms of amyloid fibril¹ and is universally present in amyloid deposits, including the cerebral amyloid of Alzheimer's disease². Although SAP is not required for amyloid fibrillogenesis *in vitro*, it may protect the fibrils from degradation *in vivo*³. SAP is also the major DNA- and chromatin-binding protein of plasma^{4,5}, and other autologous ligands for SAP include fibronectin, C4-binding protein⁶ and glycosaminoglycans⁷. Binding to all these ligands, and to amyloid fibrils, is absolutely calcium-dependent at physiological pH and ionic strength. In addition, SAP is a calcium-dependent lectin, the best-characterized ligand of which is the 4,6-cyclic pyruvate acetal of β -D-galactose⁸, and this can inhibit all other known calcium-dependent interactions of SAP. Finally, SAP is a normal tissue matrix constituent associated with elastic fibres⁹ and the glomerular basement membrane¹⁰.

Human SAP shows no polymorphism or heterogeneity of its protein or its glycan¹¹ and no occurrence of human SAP deficiency has yet been described, suggesting that the molecule has important functions. Furthermore, SAP and C-reactive protein (CRP), the classical acute-phase protein with which it shares more than 50% sequence identity, belong to the pentraxin family of plasma proteins which have been stably conserved throughout vertebrate evolution^{12,13}. The structure and structure-function relationships of SAP are thus of considerable fundamental interest, in addition to their clinical importance, which arises from

the new information provided by use of radio-labelled SAP as a specific quantitative *in vivo* tracer for amyloid deposits¹⁴.

Recently, we reported the crystallization of SAP in conditions that induce a reversible dissociation of the decamer into a pentameric form¹⁵. We now report the X-ray analysis of the crystals at 2 Å resolution, defining the complete three-dimensional structure of the pentamer. This human plasma protein has a tertiary fold which resembles that of the legume lectins concanavalin A and pea lectin. There are two calcium sites and these are shown to be involved in binding of carbohydrate and the synthetic ligand phosphoethanolamine. However, the calcium sites in SAP and the calcium and manganese sites of concanavalin A differ in their relationship to the common topology. The SAP pentamer shows nearly perfect 5-fold symmetry and sequence comparisons suggest that very similar interactions are retained in CRP pentamers.

Three-dimensional structure

Serum amyloid P component was isolated at greater than 99% purity from pooled human ascites and pleural effusion fluids¹⁶. The crystals obtained at pH 5.5 in a medium containing calcium and acetate ions as described previously¹⁵ were of space group P2₁ and cell dimensions $a = 68.9$ Å, $b = 99.3$ Å, $c = 96.7$ Å and $\beta = 95.9^\circ$. The initial model of pentameric SAP was based on X-ray analysis at 2.8 Å resolution using the multiple isomorphous replacement (MIR) technique followed by solvent flattening¹⁷ and 5-fold molecular averaging¹⁸. The refinement statistics are shown in Table 1, the legend of which gives further details of the X-ray analysis. The final model was refined at 2.0 Å resolution to an agreement (R) value of 0.18 to give clear electron density for all residues of the five subunits (see Fig. 1a).

The SAP pentamer consists of five subunits of 204 amino-acid residues, each with a closely similar three-dimensional structure constructed from antiparallel β -strands (A–O) arranged in two sheets (Fig. 2a). The tertiary fold can be envisaged as a jellyroll

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TABLE 1 Structure determination of serum amyloid P component

Derivative	K ₂ PtI ₆	Na ₃ PO ₄ (WO ₃) ₁₂	Th(NO ₃) ₄	Th(NO ₃) ₄	Ce(SO ₄) ₂	K ₂ AuCl ₄	UO ₂ SO ₄	AgNO ₃	Na ₂ WO ₄
Concentration (mM)	5.0	5.0	4.5	4.5	17.0	2.5	25.0	5.0	11.0
Soaking time (h) at 20 °C	24.0	60.0	28.0	28.0	48.0	1.0	24.0	24.0	19.0 (4 °C)
Collection device	F	F	F	F	F	F	X	I	D
Resolution (Å)	3.5	3.5	4.1	2.8	3.0	3.5	3.2	3.4	6.0
Merging <i>R</i> factor	0.084	0.069	0.062	0.049	0.101	0.110	0.045	0.118	0.075
Isomorphous <i>R</i> factor	0.317	0.237	0.203	0.278	0.222	0.310	0.251	0.165	0.225
% Complete	88.3	80.3	91.7	85.8	76.7	71.1	81.0	93.4	97.1
Number of sites	28	21	17	14	27	22	22	26	—
Phasing power	1.00	1.15	1.43	2.02 (4.1–2.8 Å)	1.02	1.55	1.29	1.04	—

Heavy-atom derivatives used for multiple isomorphous replacement and data collection statistics are shown. Native data were collected from one crystal on a Hilger-Watts 290 4-circle diffractometer with $R_{\text{sym}} = 4.5\%$ for 4,112 independent reflections to 5.6 Å. High-resolution native data were also collected on film at the Synchrotron Radiation Source at Daresbury Laboratory (wavelength 1.468 Å) using three crystals ($R_{\text{sym}} = 7.7\%$ for 78,951 unique reflections to 2.02 Å). The final merged data set comprised low-resolution data from the diffractometer and high-resolution data from the synchrotron (93.6% complete to 2 Å). The major heavy-atom sites were determined from inspection of difference Patterson functions and cross-phase difference Fourier transforms. A multiple isomorphous replacement (MIR) map was calculated, solvent-flattened and averaged. Phases from both maps were used in cross-phase difference Fourier transforms to determine those minor sites related by the 5-fold axis to the major sites. All sites were initially refined with the VECREF program (I.J.T.) to eliminate spurious sites and then with PHARE. A final MIR map was calculated to 2.8 Å and the phasing analysis gave a figure of merit of 0.61. This map was solvent flattened¹⁷, and phases were calculated and recombined with the MIR phases to give a combined figure of merit of 0.85. The solvent-flattened map was then averaged using the PSAVER program (I.J.T.) and the envelope determined in the solvent flattening. The polypeptide of the β -sheet and helix were well defined. Derivative data were also scaled to native data collected from three crystals on a FAST area detector mounted on a CuK α microfocus tube (800W) ($R_{\text{sym}} = 10.0\%$ for 30,996 independent reflections to 2.8 Å). Heavy-atom sites were determined independently from those in Table 1 and were refined using MLPARE. The resulting electron density map was solvent-flattened and averaged. The map was similar to that used for the refinement. Problems in obtaining an interpretable map arose because not all substituted sites related by the non-crystallographic symmetry were present on early cross-phase difference Fourier transforms because of poor phasing from the derivatives available at the time. Two of the three derivatives with the highest phasing power were the last to be collected. These extra derivatives allowed minor sites related to the major sites by the 5-fold axis to be determined in the early derivatives, as well as multiple occupancy in some sites previously thought to be singly occupied. For example, the dominant thorium nitrate derivative formed a complex with 11 thorium atoms comprising two octahedra, each with edges of ~ 4 Å and sharing an apex. The direction of the 5-fold axis was determined from a self-rotation function¹⁵, and the approximate position of the molecule on this axis was found by positioning a pentagonal prism in a low-resolution MIR map. A six-dimensional search (three rotations and three translations) for a best correlation in the map for a given rotational operation was performed using LOCROT (I.J.T.). The parameters were then refined with the density correlation programs of Bricogne¹⁸. One averaged subunit of the electron density map obtained from the solvent flattening was displayed in FRODO³⁵ on an Evans and Sutherland PS390. The sequence could be assigned from the position of the disulphide bridge and the putative calcium-binding site for all residues in the sheets and helix. The resolution was extended with simulated annealing using non-crystallographic restraints in XPLOR³⁶ and rebuilding into electron density maps (coefficients $2F_o - F_c$, $F_o - F_c$) calculated from phases that had been combined with the MIR phases until all residues had been inserted. The resolution was then extended to 2 Å and 10 calcium ions, 4 acetate ions and 879 water molecules were added. Least-squares refinement in RESTRAIN³⁷ gave a crystallographic *R*-factor of 0.18 for all 78,910 reflections in the resolution range 8–2 Å. The r.m.s. deviations from stereochemical ideality are 0.17 Å for bond distances and 3.45° for bond angles. The average isotropic *B* values are 22.9 Å² for protein and 37.7 Å² for solvent molecules. There were no residues in the disallowed region of a Ramachandran plot³⁸. The CCP4 suite of programs (SERC, Daresbury) has been used for all crystallographic calculations, except the structural refinement.

$$\begin{aligned} \text{Merging } R \text{ factor} & \quad \frac{\sum_{hkl} \sum_{i=1}^n |I(hkl)_i - \bar{I}(hkl)|}{\sum_{hkl} \sum_{i=1}^n I(hkl)_i} \\ \text{Isomorphous } R \text{ factor} & \quad \frac{\sum_{hkl} |F_{\text{deriv}}(hkl) - F_{\text{nat}}(hkl)|}{\sum_{hkl} |F_{\text{deriv}}(hkl)| + \sum_{hkl} |F_{\text{nat}}(hkl)|} \\ \text{Phasing power} & \quad \left[\frac{\sum_{hkl} F_{\text{heavy}}^2(hkl)}{\sum_{hkl} e^2(hkl)} \right]^{1/2} \end{aligned}$$

D, Enraf-Nonius CAD-4 diffractometer mounted on a 1,500 W sealed tube; F, Enraf-Nonius FAST area-detector mounted on a CuK α microfocus tube (800 W); X, Siemens Xentronics area-detector mounted on a Siemens XP18 generator; I, MAR research imaging plate mounted on a Siemens XP18 generator.

of strands ABCDKLNO, elaborated by the addition of three further antiparallel strands (EFG and HIJ) forming a β -meander at the same end of each of the sheets to give the topology +11–9+7–1–1–1–3+1+1+5–9–1+12–13. In this arrangement, strands A and M are both hydrogen-bonded to strand L (see Fig. 3). The disulphide between Cys 36 and Cys 95 links the two adjacent strands (L and C) of one β -sheet. A long α -helix between strands L and M is folded on top of this β -sheet. There is also an *N*-linked oligosaccharide at Asn 32 on this sheet; only one saccharide residue is visible in the electron density map.

The hydrophobic core between the two sheets is comprised mainly of tryptophan, tyrosine, phenylalanine and leucine. The core is closed off by two β -arches between the two sheets; strands joining B to C and J to K are hydrogen-bonded and antiparallel, an arrangement characteristic of proteins derived from a jellyroll motif. One end of the core, formed by 11 residues at the amino-

terminus and those in the N and O strands at the carboxy terminus (Figs 2, 3), has hydrophobic residues accessible to solvent. The other end of the core is involved in interactions with a neighbouring promoter (see below) and so is inaccessible to solvent.

Tertiary fold comparisons

The similarities of the amino-acid sequences of SAP, human CRP, CRP of the arachnid *Limulus polyphemus* (the horseshoe crab) and hamster SAP suggest that they may have similar three-dimensional structures. Comparative modelling¹⁹ shows that these pentraxins can have equivalent antiparallel structures, with insertions and deletions in the loops between the β -strands and α -helices (N.S., H.E.W. and T.L.B., unpublished results). Figure 4 shows that most solvent-inaccessible aromatic side chains are conserved to give compact hydrophobic cores in all members of the family.

The jellyroll topology of the pentraxins is reminiscent of that of the picornavirus coat proteins, which also have pentameric structures. However, pentraxins most closely resemble legume lectins such as concanavalin A²⁰ and pea lectin²¹ (Fig. 2a). In each case, the arrangement of strands is identical, but the N and C termini are in different positions (Fig. 3). In pea lectin, the N terminus is at strand M and the C terminus at strand L (labelling of the topologically equivalent strands follows those in SAP as shown in Fig. 3). Strands A and M are both hydrogen-bonded to strand L in a similar manner to those in SAP. In the three-dimensional structure of concanavalin A, the N terminus is at strand E and the C terminus at the end of strand D owing to a post-translational cleavage which follows ligation of the true termini between strands L and M²². Pea lectin is additionally cleaved at the loop connecting strands I and J.

Alignment of sequences (Fig. 4) on the basis of topologically equivalent features of the three-dimensional structures²³ shows that helices occupy different positions in the pentraxins and legume lectins and that the amino-acid sequences of the two families have identities of only ~11%. The two main helices in SAP occur before and after strand L, whereas the helices in the legume occur at the C terminus of strand J. There is a long insertion between the end of the helix after strand D and the beginning of strand E in the lectins relative to the pentraxins. Strands G, H and I, together with the type IV β -hairpin between H and I, are identical in both SAP and pea lectin. The so-called pentraxin octapeptide signature sequence, HXCXS/TWXS, is in this region, but this is not conserved in the legume lectins.

Structure of pentamer and decamer

Figure 2b shows the structure of the pentamer. The five subunits are arranged in a ring with a hole 20 Å in diameter and 35 Å deep at the centre. The two layers of β -strands in each subunit are in planes normal to the 5-fold axis. Strands G, I and J of one protomer interact with strand N and loops between strands A and B, C and D, G and H and K and L of an adjacent protomer (Fig. 2b). When SAP is overlaid on the pea lectin, strand J does not give as good a fit as other strands. In SAP this strand has moved to provide inter-protomer contacts, with all residues between Pro 113 and Leu 119 being involved. The subunit interactions consist of hydrogen bonds between main-chain peptide groups, three salt bridges and some hydrophobic contacts, in contrast to other pentameric systems where there are often inter-subunit β -sheets. The surface area of the protomer that is buried on formation of the pentamer is 410.5 Å², comprising 15.4% of the total surface of the protomer. Figure 4 shows the residues involved in these extensive interactions and which account for the considerable stability of the SAP pentamer.

In contrast, the SAP decamer is readily dissociated by reducing the pH to 5.5. The simplest explanation for this is that the decamer is stabilized by ionic interactions involving carboxylate and/or imidazole groups. Electron microscopy has clearly shown that the SAP pentamers are packed face-to-face²⁴ and it seems probable that the faces in contact are those carrying the α -helix, as we show (see below) that the calcium-dependent ligand-binding sites are on the other face and such binding is exhibited by the decamer. Furthermore, adjacent sites are accessible to chymotryptic cleavage in the decamer in the absence of calcium ions. It is not clear which groups are involved in the

decamer stabilization, although Glu 167, positioned on the helix, is a likely candidate. A compact decamer can be modelled if the pentamer 5-fold axes are in line but the subunits are out of step, allowing the helices from one layer to pack between those of the opposite layer. Structure analysis of a crystal form produced at neutral pH with 10 protomers in the asymmetric unit is in progress.

The models of CRP and hamster SAP demonstrate that the pentamers can have very similar inter-subunit interactions. *Limulus* CRP is hexameric²⁵ and we have also constructed this arrangement of protomers by operating with 6-fold axis placed slightly farther from the subunit than the 5-fold axis of SAP.

Calcium-binding site

In serum amyloid P component, we have identified two large spheres of density which are too heavy to be oxygen atoms and are in positions that imply the presence of calcium ions, between 4.0 and 4.3 Å apart in the five subunits and bridged by common side chains. Calcium ion 1 is coordinated to the side chains of Asp 58, Asn 59, Glu 136, Asp 138 and the main-chain carbonyl

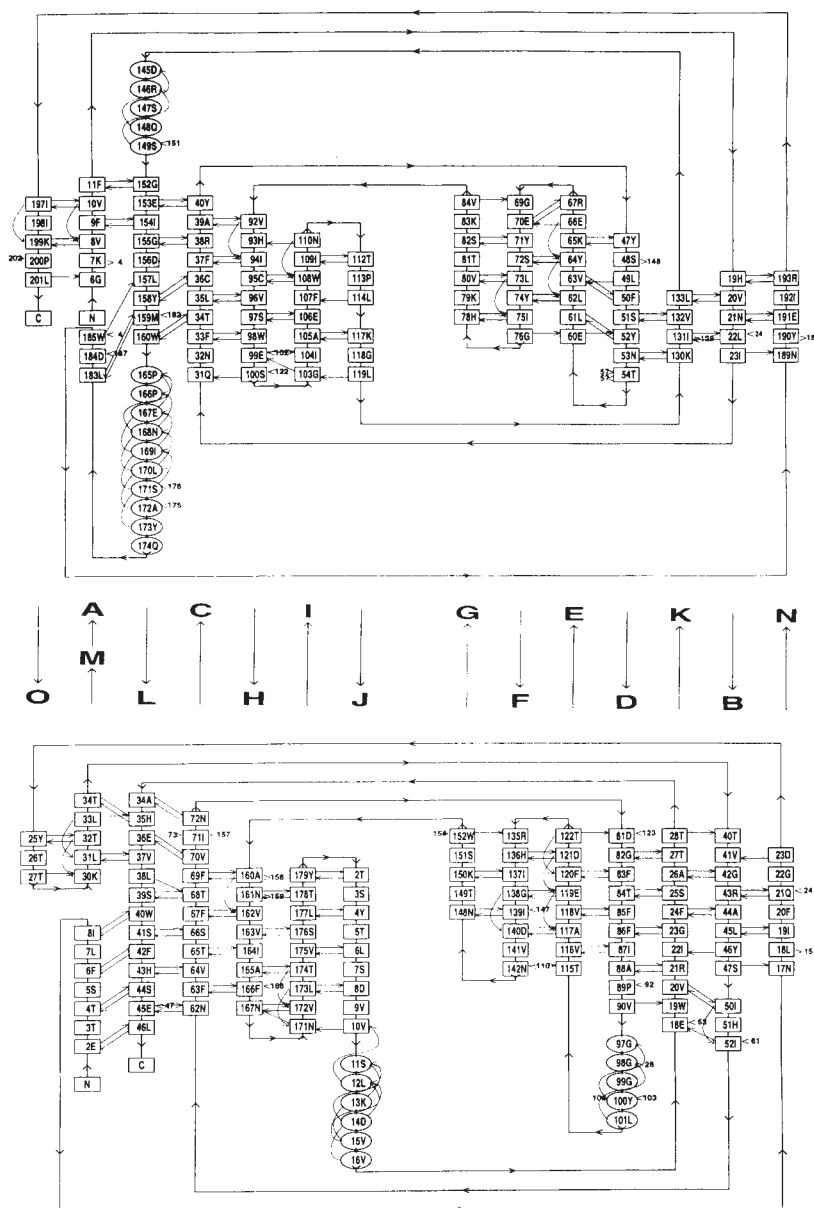


FIG. 3 Hydrogen-bonding diagrams generated by HERA⁴⁰ for SAP (above) and pea lectin (Brookhaven code 2LTN) (below). The secondary structural elements were assigned according to the criteria defined by Kabsch and Sander⁴¹.

of Gln 137 (Fig. 5a). Unlike many calcium-binding sites in proteins, such as parvalbumin, the coordinating residues come from different parts of the sequence. This is achieved by a distortion at the start of strand E carrying residues Asp 58 and Asn 59 and the region containing Glu 136 and Asp 138 looping over towards calcium 1 (Fig. 5a). The calcium ligation is likely to be an important local structural determinant. The seventh coordination site is occupied by a ligand that has the appearance of an acetate ion from the crystallization buffer in protomers 1, 2, 4 and 5,

but in protomer 3 this position is filled by the side chain of Glu 167 of an adjacent molecule in the crystal lattice. Glu 136, Asp 138 and the acetate/lattice contact form a bridge to a second, more loosely bound calcium ion (calcium 2). The coordination of calcium 2 is completed by Gln 148 and two water molecules. In a cross-phase difference Fourier transform of cerium sulphate soaked crystals, we find that calcium 2 is displaced by a cerium ion. Calcium 2 is also removed when the crystals are soaked in calcium-free buffers. These observations are consistent

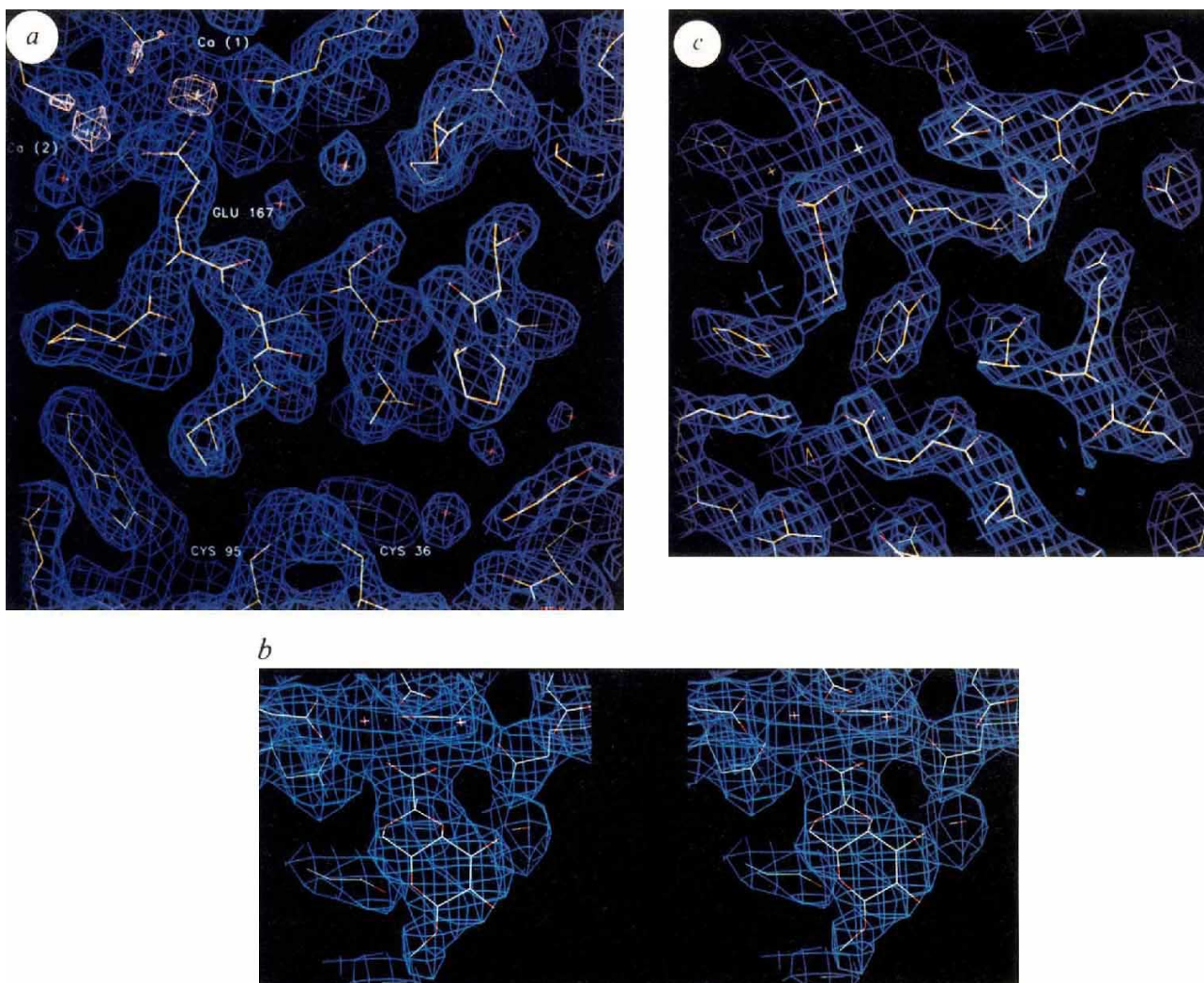


FIG. 1 Final refined electron density of SAP. *a*, Refined electron density map ($2F_o - F_c$) calculated at 2 Å resolution and contoured at 1 r.m.s. This shows the SAP helix packing against the β -pleated sheets in the region of the disulphide bridge Cys 36–Cys 95. The top left hand of the diagram shows a region where the side chain Glu 167 makes contact with the calcium-binding site of a symmetry-related molecule. The region of the calcium ions is contoured at 5 r.m.s., showing that the glutamate bridges the two ions in a similar manner to the acetate ion. *b*, MO β DG complexed with SAP contoured at 1 r.m.s. electron density level at 2.9 Å resolution. Crystals of SAP prepared by the batch method¹⁵ in the presence of MO β DG were isomorphous with crystals of native SAP and had cell dimensions $a=69.06$ Å, $b=99.3$ Å, $c=96.75$ Å and $\beta=95.84^\circ$. Data were collected on a MAR imaging plate system mounted on a Siemens XP-18 rotating anode and a final R_{merge} of 5.3% was obtained. Data were 95.8% complete to 2.9 Å resolution. A final crystallographic R factor of 0.197 for all reflections in the range 10–2.9 Å was obtained after refinement in XPLOR without non-crystallographic restraints. The success of this experiment depended on soaking in an excess of the sugar so as to displace the acetate ion which otherwise occupies the same position at the calcium-binding site. The

MO β DG is well defined only in promoter 3, shown here, where it is involved in a crystal contact; there is density consistent with the same binding at other subunits. *c*, Phosphoethanolamine (PE) complexed with SAP contoured at 1 r.m.s. electron density level at 2.9 Å resolution. Crystals of SAP prepared by the batch method¹⁵ in the presence of 9 mM PE were not isomorphous with crystals of native SAP and had cell dimensions $a=67.05$ Å, $b=103.43$ Å, $c=102.43$ Å and $\beta=95.73^\circ$, in space group $P2_1$. Data were collected on a MAR imaging plate system mounted on a Siemens XP-18 rotating anode and a final R_{merge} of 6.4% was obtained. Data were 95.5% complete to 2.9 Å resolution. In the initial refinement, the SAP pentamer was treated as a single rigid body and an R factor of 0.33 was obtained for the 3,513 reflections at 30–6 Å. The five subunits were then treated as individual rigid bodies and refinement produced an R factor of 0.26 for the 11,628 reflections at 18–4 Å. The final crystallographic R factor of 0.20 for all reflections in the range 10–2.9 Å was obtained after refinement in XPLOR; non-crystallographic symmetry restraints were not applied. Coordinates have been deposited with the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, New York 11973, USA.

with the more solvent-accessible position and fewer protein ligands of calcium 2.

Residues that provide ligands to the calcium ions are conserved at the start of strand E carrying residues Asp 58 and Asn 59 and the region containing Glu 136 and Asp 138 looping over towards calcium 1 (Fig. 5a). The calcium ligation is likely to be an important local structural determinant. The seventh coordination site is occupied by a ligand that has the appearance of an acetate ion from the crystallization buffer in protomers 1, 2, 4 and 5, but in protomer 3 this position is filled by the side chain of Glu 167 of an adjacent molecule in the crystal lattice. Glu 136, Asp 138 and the acetate/lattice contact form a bridge to a second, more loosely bound calcium ion (calcium 2). The coordination of calcium 2 is completed by Gln 148 and two water molecules. In a cross-phase difference Fourier transform of cerium sulphate soaked crystals, we find that calcium 2 is displaced by a cerium ion. Calcium 2 is also removed when the crystals are soaked in calcium-free buffers. These observations are consistent with the more solvent-accessible position and fewer protein ligands of calcium 2.

Residues that provide ligands to the calcium ions are conserved in all SAPs, but although Asp 58 is found in hamster SAP, human CRP and *Limulus* CRP, it varies in other CRPs. Nevertheless, the general organization of the site is probably retained. The disposition of these groups on surface loops where sequence differences accumulate could explain the considerable change in calcium affinity between CRP and SAP. In CRP there is evidence that both calciums bind with the same affinity at neutral pH²⁶, whereas in SAP our results show that calcium 2 has fewer protein ligands and can be preferentially unloaded. The tighter calcium binding and equivalence of the sites in CRP could also be due to the substitution of Asp 145 in SAP by glutamate in CRP. The longer side chain in CRP would permit a full complement of protein ligands to calcium site 2.

The existence of two metal ions bridged by protein ligands is reminiscent of concanavalin A. However, although the metal-binding sites in the legume lectins are on the same face of the

protein, they are at a different position, between strands E and F, compared with D and E on SAP. There are no ligands of the two calcium ions that are topologically equivalent to those for the calcium and manganese of the lectins. Thus, although there is evidence for divergent evolution of the protein folds of the plant lectins and the pentraxins, this is not supported by a conservation of similar metal-binding sites.

Ligand binding

SAP binds to methyl 4,6-*O*-(1-carboxyethylidene)- β -D-galactopyranoside (MO β DG), which is not recognized by CRP. Figure 1b shows the electron density for MO β DG complexed with SAP. The sugar derivative binds directly through the acidic group to the two calcium ions in a similar way to the acetate that it replaces. The other interactions include two hydrogen bonds formed between the 4, 6 oxygen atoms of the ring and the amide nitrogen atoms of Gln 148 and Asn 59, respectively, each of which bind to the calcium ions through their amide oxygens (Fig. 5b). Thus, the role of calcium is not to bind the galactopyranoside ring directly, but rather to mediate its binding by orienting side-chain amides in a way that resembles saccharide binding in lectins. There is only one hydrogen bond to the galactopyranoside ring (Fig. 5b). Thus, it is the methyl 4,6-*O*-(1-carboxyethylidene) ring that forms the main interactions with the protein, explaining why neither the non-cyclic acetal of MO β DG nor the simple monosaccharides bind (refs 3, 8 and M.B.P. *et al.*, manuscript in preparation). The *R*-isomer bridge methyl group points into a hydrophobic pocket formed by Leu 62, Tyr 64 and Tyr 74. The differences in the hydrophobic pocket and the ligand distribution at the calciums in CRP may explain the poor binding to MO β DG. It is probable that this site is involved in binding to amyloid fibrils, and its specificity will be discussed elsewhere (M.B.P. *et al.*, manuscript in preparation).

The highest-affinity interaction of human CRP is its calcium-dependent binding to phosphocholine (PC). Studies on human CRP^{27,28} have implicated amino-acid residues 50–60 in the ability

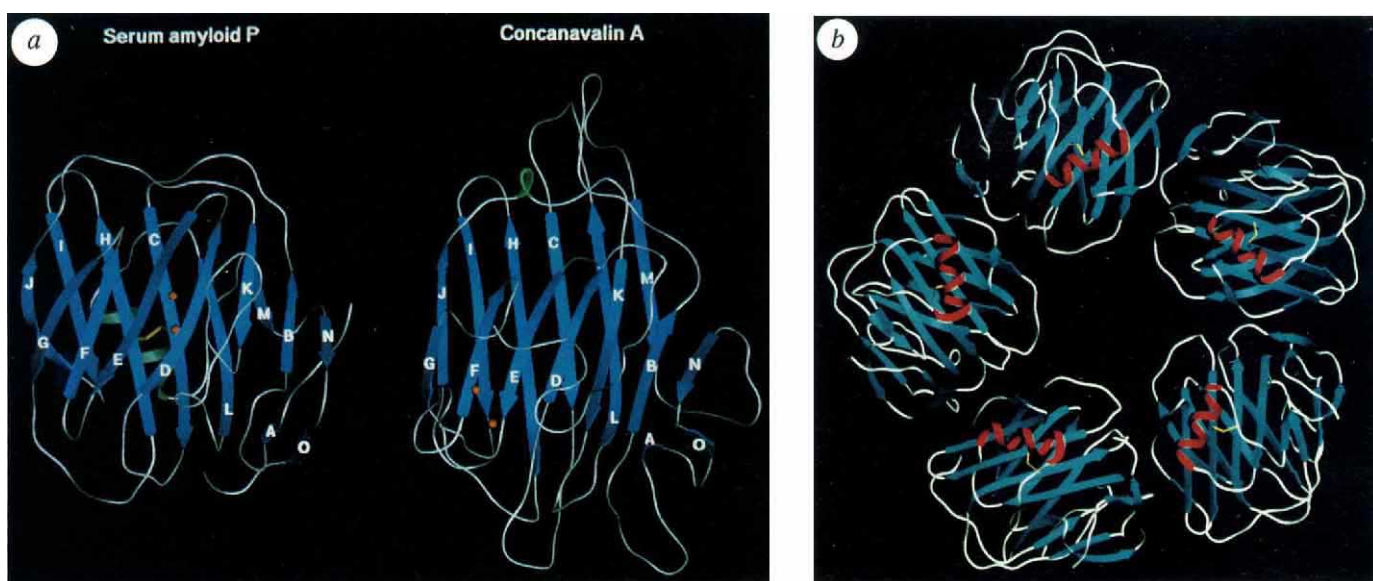


FIG. 2 Ribbon drawings generated with Setor³⁹ with β -strands shown as blue arrows, helices as ribbons and loops as cords. a, The topology of SAP compared with the legume lectin concanavalin A (Con A). The strands are labelled from the N terminus of SAP, ABCDEFGHIJKLMNO, with structurally equivalent strands in Con A labelled similarly. The positions of the N and C termini in Con A are altered with respect to the nascent protein by ligation of the origin N and C termini and proteolytic cleavage between strands D and E. The two structures can be superim-

posed by a least-squares fit using the C α positions of the two sets of antiparallel sheets to produce an r.m.s. deviation of 2.4 Å. There is a greater angle between these sheets in SAP than in Con A. b, Structure of the pentamer of SAP viewed along the non-crystallographic 5-fold axis of symmetry. The pairwise r.m.s. deviations for all main-chain atoms range from 0.19 Å between protomers 1 and 5 to 0.24 Å between protomers 3 and 4.

to bind PC, and more recent mutagenesis experiments²⁹ have identified Lys 55 and Arg 56 as key residues. The native SAP structure shows that Asp 58 and Asn 59 from this loop are involved in coordinating one of the two calcium ions. Human SAP, in contrast, does not bind to PC, although in common with CRP it does bind to phosphoethanolamine (PE). The electron density maps for SAP co-crystallized with PE (Fig. 1c) show a major site in all subunits, which coincides with that which binds acetate ions and MO β DG and indicates a direct interaction between the phosphate group and both calcium ions. In contrast to MO β DG, this interaction with PE displaces Glu 167 from its intermolecular interaction with the calcium ions of protomer 3, explaining the observed disturbance of crystal packing. Binding of MO β DG or PE in the common site probably stabilizes the whole calcium-binding region, including Asn 59 which binds MO β DG and calcium and Asp 58 which binds calcium. Both PE and PC probably bind at the calcium ion in CRP.

SAP aggregates rapidly in neutral solutions in the presence of

calcium ions, presumably through intermolecular interactions involving the surface of the decamer with the exposed calcium-binding site. This is consistent with the observation that this interaction can be inhibited by MO β DG³⁰ and by PE (M.B.P. *et al.*, manuscript in preparation). Similarly, they compete for the calcium site with Glu 167, which is an important lattice contact in the pentamer crystals.

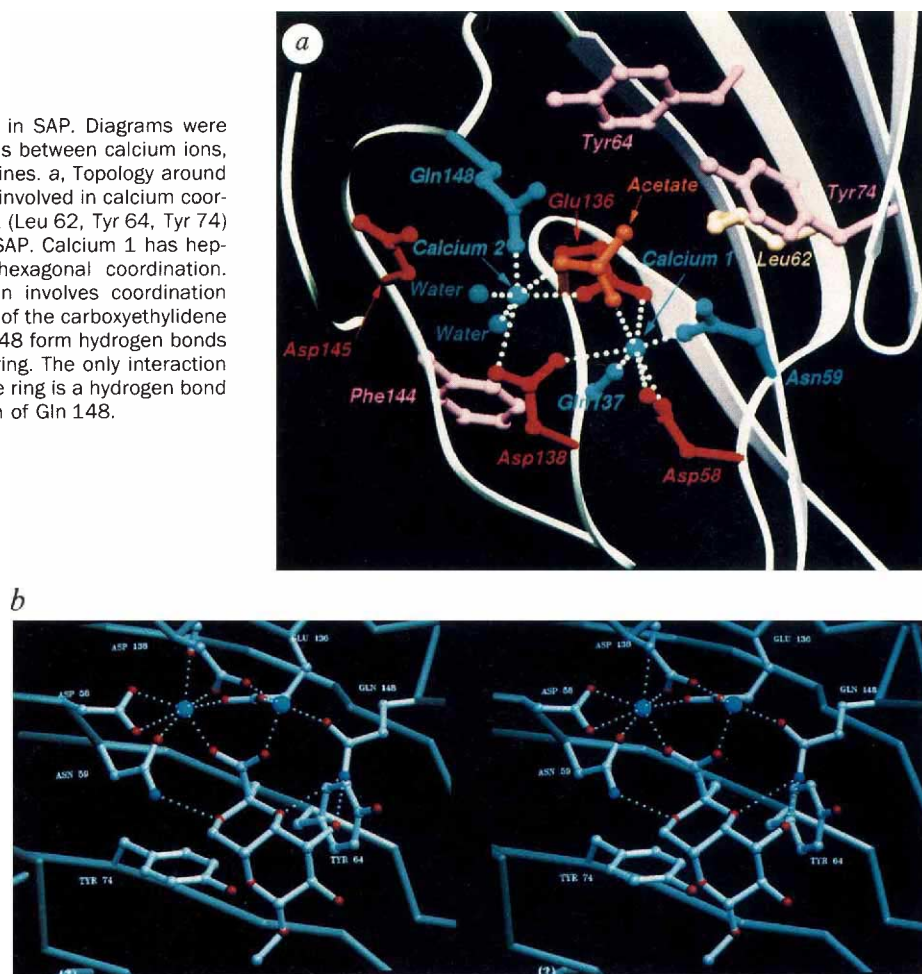
In the presence of calcium, SAP also binds polyanions such as heparan sulphate, dermatan sulphate⁷ and DNA⁴. It has been suggested that the DNA interaction involves a decapeptide around Arg 120, with some resemblance to certain histone sequences, and for which a helical structure was proposed³¹. However, in human SAP this region is not helical and other SAPs do not have arginine at this position. Nevertheless, it does have affinity for multivalent anions, as demonstrated by the binding of phosphotungstate at this site in the heavy-atom isomorphous derivative (see Table 1). There are several basic patches in SAP, for example Arg 120, Arg 77, His 78, Lys 79 and Arg 57, which are on the same surface as the calcium- and



FIG. 4 Alignment of the sequences of the pentraxins (one protomer sapm and one pentamer sapp) of SAP, human C-reactive protein (hcrp), hamster SAP (female hamster protein) (1fhp) and *Limulus* CRP (1lim) with the sequences of legume lectins, concanavalin A (3cna) and pea lectin (2ltu and 1lte). These alignments are based on comparing the three-dimensional structure of SAP with those of the legume lectins²³. Key to characters in the alignments⁴²: solvent inaccessible, upper case

letter; solvent accessible, lower case letter; positive ϕ , italic letter; cis-peptide, breve over letter; hydrogen bond to other side chain, tilde over letter; hydrogen bond to main-chain amide, bold letter; hydrogen bond to main-chain carbonyl, letter underlined; disulphide bond, cedilla below letter; * metal-binding sites in SAP and Con A; | residues of SAP involved in inter-protomer contacts.

FIG. 5 The calcium- and ligand-binding sites in SAP. Diagrams were generated using Setor³⁹. Important interactions between calcium ions, ligands and the protein are shown by dotted lines. a, Topology around the calcium-binding site showing the residues involved in calcium coordination and those in the hydrophobic pocket (Leu 62, Tyr 64, Tyr 74) and the cleavage site (Phe 144, Asp 145) in SAP. Calcium 1 has heptagonal coordination, and calcium 2 has hexagonal coordination. b, MO β DG-binding site. The main interaction involves coordination between the calcium ions and the carboxylate of the carboxyethylidene ring. The amide nitrogens of Asn 59 and Gln 148 form hydrogen bonds to the 4, 6 oxygens of the carboxyethylidene ring. The only interaction between the protein and the galactopyranoside ring is a hydrogen bond between the 3 oxygen and the amide nitrogen of Gln 148.



PE-binding sites. It seems more likely that the phosphate backbone of DNA and the sulphated polysaccharides bind both at the calciums and at the basic sites, possibly on more than one subunit simultaneously. These extensive interactions probably account for the ability of SAP to displace from DNA the H1-type histones in chromatin⁵. The sequence differences in the basic regions and in the calcium-binding region could explain the variable affinity for DNA shown by different pentraxins. Human CRP, for instance, binds DNA only at low, non-physiological ionic strength. Low concentrations of both PE and MO β DG enhance the binding of SAP to DNA and both compete with the interaction at high concentration (M.B.P. *et al.*, manuscript in preparation). Similar effects are observed for SAP binding to immobilized PE (M.B.P. *et al.*, manuscript in preparation). As we can find no evidence for a second binding site for PE or MO β DG in the present experimental conditions, these effects may be mediated through inter-subunit interactions.

Ligands for SAP, such as PE in phosphatidylethanolamine and MO β DG-like sugars³², are common in microorganisms. In this respect it resembles CRP¹³ and mannose-binding protein³³, a plasma C-type lectin involved in innate resistance to infection; these recognize widely distributed microbial epitopes, suggesting that SAP, too, may participate in host defence.

Proteinase resistance and amyloidosis

SAP and CRP are both remarkably resistant to proteolytic degradation in the presence of calcium. In contrast, in the absence of calcium both are cleaved by some enzymes, particularly α -chymotrypsin and pronase^{26,34}. Although this cleavage does not cause fragmentation of either the whole molecule or the individual subunits under non-denaturing conditions, it does cause loss of calcium-binding activity by the pentraxins and

abolishes their capacity for calcium-dependent ligand binding. It is therefore of interest that the major site of cleavage of SAP is at residues 144–145 whereas in CRP it is at residues 146–147 (pronase) or 145–146 (Nagarse protease). This is part of a loop held in place by calcium ligation which, in the calcium-free form, may be only loosely associated with the body of the protein and, therefore, susceptible to proteolysis. Most loops of SAP are held close to the body of the pentamer and this makes them less easily accessible to the active sites of proteolytic enzymes.

Resistance to proteinase digestion is likely to be an important aspect of the normal function of SAP, and may also contribute to the persistence of amyloid deposits. The SAP normally associated with the glomerular basement membrane and the surface of elastic fibre microfibrils^{9,10} may protect these components of the extracellular matrix from inappropriate degradation. On the other hand, amyloid fibrils are abnormal extracellular structures which should be recognized and degraded, but which nevertheless persist *in vivo*. In this pathological situation, the binding of SAP to amyloid fibrils may be responsible. Protection could result simply from coating by SAP, which is completely unaltered with respect to its normal circulating form¹¹, and which would therefore not be expected to trigger macrophage activation or phagocytosis. However, the proteinase resistance of SAP itself may be a significant factor. Availability of the complete high-resolution structure of SAP and its ligand-binding site now offer the opportunity for direct modelling of competitive inhibitors of SAP binding and for producing binding-site homologues, either of which could be used as drugs to displace SAP from amyloid deposits *in vivo*. This would open up new avenues for treatment of amyloidosis, enabling the body to mobilize and degrade the fibrils which may otherwise be inappropriately protected by SAP. □

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LETTERS TO NATURE

Transition between spiral and target states in Rayleigh–Bénard convection

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RAYLEIGH–BÉNARD convection^{1–4}, which occurs when a shallow fluid layer is heated from below, is commonly regarded as a paradigm for pattern formation under non-equilibrium conditions. The formation of hexagonal arrays of Bénard cells is well known, but more complex patterns such as targets⁵ and spirals^{5–11} have also been reported. Similar patterns have been seen in electrohydrodynamical convection^{12,13}, oscillatory chemical reactions^{14–19} and biological systems^{19,20}. In general, the spiral and target states are found for different experimental conditions. Here we report the observation of a continuous transition between states containing many spirals and many targets, in a fluid undergoing Rayleigh–Bénard convection near the gas–liquid critical point. Whether spirals or targets are observed depends on the Prandtl number, the ratio between the thermal and viscous timescales in the fluid. Neither of these states seems to be predicted by the hydrodynamic equations that describe the fluid motions^{1–4, 21}. The fact that the transformation of one pattern into the other is continuous, and that under some conditions they can coexist, suggests that they may be generated by the same or a similar mechanism.

We studied Rayleigh–Bénard (RB) convection in SF₆ near its thermodynamic critical point (the critical temperature, pressure and density are $T_c = 318.7$ K, $p_c = 37.8$ bar and $\rho_c = 0.73$ g cm⁻³ respectively)⁵. The fluid was enclosed in a stainless steel vessel having a sapphire window 19 mm thick and 102 mm in diameter. A nickel-plated copper bottom-plate (to which a thermofoil heater was attached) sandwiched a mylar spacer. The spacer had an inner diameter $D = 30$ mm and thickness $d = 130$ μ m, giving a radial aspect ratio of $\Gamma = D/2d = 115$. The vessel was coupled by a thin tube to a small hot volume, in which pressure feedback could be performed by heating the fluid. Both vessels were enclosed in a constant-temperature bath in which the long-term stability was better than ± 1.0 mK. Long-term stability of the

bottom temperature was better than ± 0.3 mK. Pressure feedback resulted in long-term stability of ± 0.5 mbar. Shadowgraph techniques, with and without computer enhancement, were used to visualize the patterns. The cell was filled far from the critical point so that the desired density could be obtained with an accuracy of $\pm 0.25\%$ from an equation of state²².

The RB problem has two independent control parameters, namely the Rayleigh number Ra which represents the dimensionless temperature difference across the fluid and is a measure of the external stress applied to the system, and the Prandtl number Pr ^{1, 4, 21}. In the Boussinesq approximation^{1, 4, 21} (roughly speaking, temperature-independent fluid properties) the pattern selection is completely determined by the values of Ra , Pr and k , the wavenumber of the pattern^{1, 4, 21}. These patterns are summarized in what is known as the Busse balloon (the part of the Ra , Pr , k phase space bounding the stable patterns) and have been verified extensively both experimentally and theoretically^{1, 3}. Deviations from the Boussinesq approximation have been shown to influence the pattern selection near onset^{1, 4, 21, 23}. Non-Boussinesq behaviour of fluids may be quantified by the parameter Q , a global dimensionless measure of the variations of the relevant fluid properties (density, viscosity, thermal conductivity and isobaric specific heat and thermal expansion) with temperature²¹. Its role for the pattern selection is commonly neglected, especially far from onset.

Recently, we showed that RB convection near T_c has the following advantage because most thermodynamic and kinetic properties of fluids have a universal power law behaviour as a function of the reduced average temperature $\tau = (\bar{T} - T_c)/T_c$: (1) the onset of convection (ΔT_0) decreases as τ is decreased, enabling convection in thin cells and thus large aspect ratios; (2) Pr can be scanned continuously from ~ 1 to practically infinity; (3) Q can be varied independently because it is a function of d , τ and ΔT . Here $\bar{T}$ and ΔT are the average temperature and the temperature difference across the fluid layer respectively⁵.

In Fig. 1a, b and c we present spiral, target and mixed states for values of the Prandtl number of 3.3, 6.6 and 3.6 respectively. For intermediate Prandtl numbers ($3 \leq Pr \leq 4$) as well as in transients, we observed both patterns simultaneously and transitions between them. On the whole, the steady state, comprising many extended patterns, remains time dependent, although single stationary spirals and targets are also present. Spiral-pattern-dominant states exist for $Pr \lesssim 3.5$, while target pattern dominant states

occur for $Pr \geq 3.5$. We found targets to exist at least for Pr up to 30. Within experimental accuracy the target-spiral transition occurs continuously without hysteresis in a finite range of Prandtl numbers above a certain value of Ra . In this range the ratio of the number of spirals to targets changes continuously with Prandtl number. The formation of both extended patterns is always defect mediated. Both patterns expand, during transients, until their growth is stopped by boundaries or neighbouring extended patterns. Targets expand either due to a focus instability or due to concentric travelling waves emitted from the centre. Spirals, however, usually grow by tip rotation. Single defects—always present in the system—do not stop the growth, but can act as initiators for spiral-to-target transitions. When the wave number is not axisymmetrically uniform, target-to-spiral transitions may also be observed. Figure 2a shows a close-up of the transition from spirals to targets and Fig. 2b presents the opposite case. In both cases a reconnection, on a timescale of the vertical diffusion time τ_v , in the vicinity of the defect core is responsible for the transition. It is caused either by the influence of nearby defects or simply by wavenumber adjustment. This matching of the local to the optimal wavenumber often causes roll bulging, pinching and bridging (see Fig. 3). The latter is reminiscent of the amplitude instability suggested by Newell *et*

*al.*²⁴ and at later times initiates the creation of the novel extended patterns. The breakup-reconnection mechanism is quite general and does not appear only in spiral-target transitions, as depicted in Fig. 4. It is important to note that in our experiments both the spiral and target patterns appear for values of $Q \lesssim O(1)$. In the many-spiral state, spirals are rarely more than single armed. Stationary and rotating spirals of either topological charge usually coexist in the cell. Quite surprisingly, they can rotate in the direction of the spiral winding or against it.

Recent experiments⁶⁻⁸ and numerical simulations⁹⁻¹¹ for single and many spirals in the cell (for $Pr \lesssim 1$) deduce that non-variational effects due to large-scale mean flow are responsible for the rotating spiral formation at these low Prandtl numbers. This means that the spatio-temporal dynamics of the pattern can no longer be described by an equation based on an extremum principle. The significance of this effect is proportional²⁵ to Pr^{-1} , and therefore, at large enough Pr we rather expect the non-Boussinesq effect (or non-variational terms due to it) to cause the pattern. As large-scale mean flow is also proportional to the fluid thickness, its influence in our experiments should even be smaller than in the experiments of refs 6-8. Side-wall forcing, also thought to be a possible mechanism^{6,7,9,10}, is not responsible for our patterns, as they nucleate in the interior of the cell, as in refs 8 and 11. Furthermore, although from a theoretical point of view $Q \sim O(1)$ is small (non-Boussinesq effects scale as

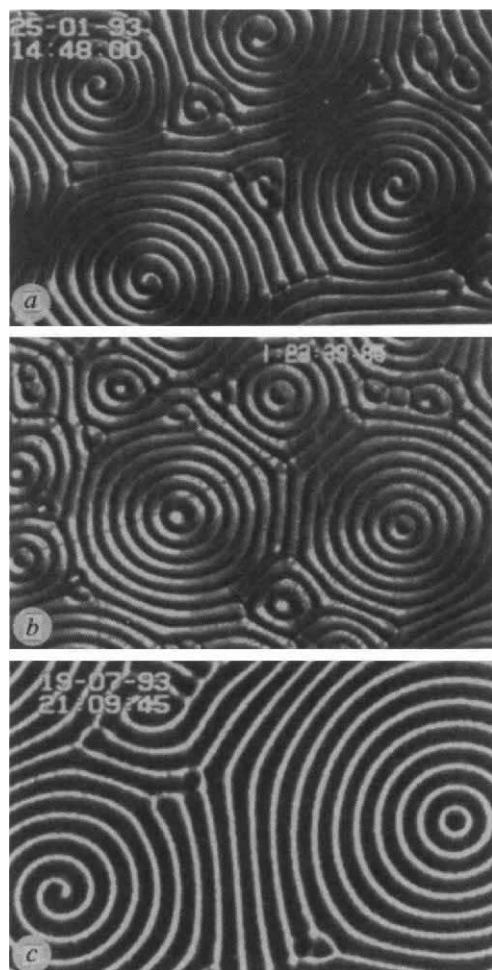


FIG. 1 Examples of high-spatial resolution shadowgraph images of the different extended patterns seen in a fraction of the cell near the centre. *a*, Spirals with $Pr=3.3$, $Ra/Ra_0=3.64$ and $Q=2.55$. *b*, Targets with $Pr=6.6$, $Ra/Ra_0=3.63$ and $Q=0.46$. *c*, A mixed spiral/target pattern state for $Pr=3.6$, $Ra/Ra_0=3.08$ and $Q=3.47$ (Ra_0 is the critical Rayleigh number for convection onset). Note that large extended patterns such as in *c* are quite robust against perturbations by defects. The horizontal size of each frame is ~ 4.5 mm.

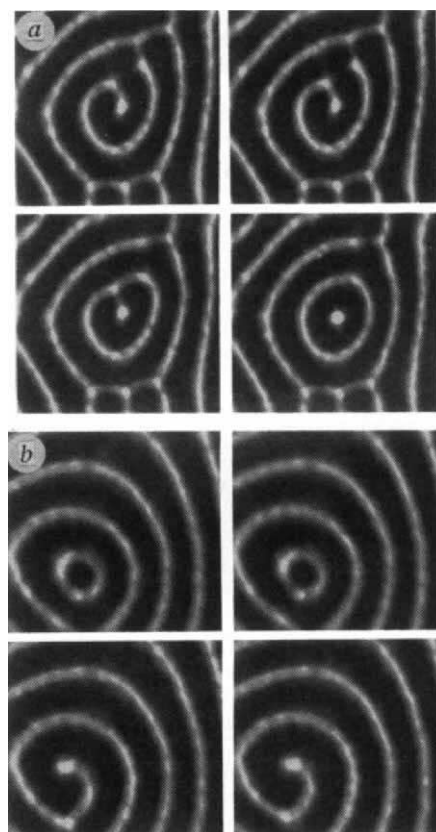


FIG. 2 *a*, The transition from spirals to targets appears at the centre of the extended pattern, by a fast reconnection of the tip to a nearby roll of the spiral. Usually this transition is initiated by nearby defects (not shown here), which cause a mismatch between the local and optimal wavenumber of the pattern. *b*, During the transition from targets to spirals, the innermost roll breaks up and then reconnects to form a spiral. Sometimes, the system breaks a roll but cannot decide whether to reconnect to form a target or a spiral. This sometimes leads to a quasiperiodic break-up reconnection (Fig. 4). Time progresses from left to right and top to bottom in steps of about one vertical diffusion time $\tau_v = 3$ s. Each frame in *a* and *b* is 0.85 and 0.75 mm wide respectively.

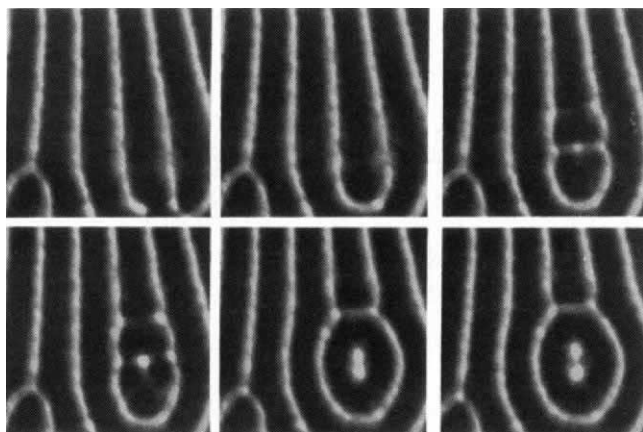


FIG. 3 Convex disclinations lead to a local wavenumber increase. Roll bulging, pinching and bridging then consecutively occur within about one $\tau_v = 3$ s. The bridge causes a target pattern to form. Each frame is ~ 0.85 mm wide. Time progresses from left to right and top to bottom.

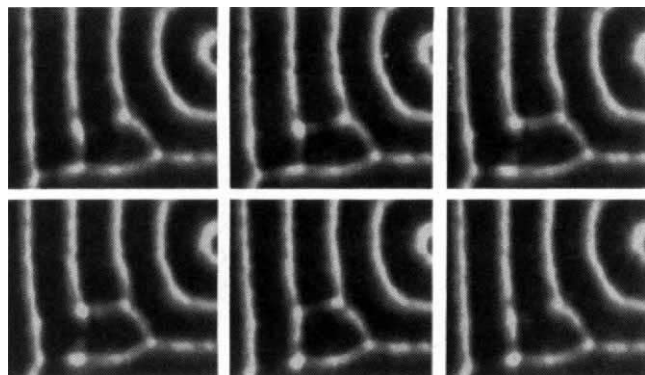


FIG. 4 Single period of a breakup and reconnection which appears during more than 20 consecutive cycles in the system. Although the quasiperiodic breakup–reconnection process shown here does not occur in the centre of the extended patterns it is generic and can also be seen in target-to-spiral transitions. This might indicate that multi-states are viable. Each frame is ~ 0.75 mm wide. Time progresses from left to right and top to bottom in steps of about one τ_v .

$6Q^2/Ra_0$ with $Ra_0 = 1,708$, where Ra_0 is the critical Rayleigh number for the onset of convection), the hexagons observed near onset²³ for such values of Q are a definite signature of the non-Boussinesq behaviour. Considering then the similarities between spirals and targets and realizing that mean flow effects are negligible at high Pr, we conclude that possibly non-Boussinesq effects are important for their formation, especially in light of previous exhaustive studies on Boussinesq convection which has not revealed them^{1, 4, 21}.

Bodenschatz *et al.*^{6, 7} observe spirals as a direct transition from hexagons when Ra is increased, whereas in our case the transition is from hexagons to straight rolls and only at higher Ra to defect mediated spiral or target formation. The latter case for spirals, however, has also recently been observed by Morris *et al.*⁸ and Xi *et al.*¹¹.

It is clear that a satisfactory explanation for these extended patterns is lacking. A more thorough investigation, singling out the possible mechanism(s), is definitely required and is currently underway. From a theoretical standpoint the question that should be addressed is how patterns of such different symmetry and topology are related. □

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Structure of the microporous titanosilicate ETS-10

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INORGANIC microporous framework solids such as zeolites are of considerable technological importance as shape-selective catalysts, ion-exchange materials and molecular sieves¹. Most microporous materials known until recently were silicates, aluminosilicates¹ or aluminophosphates^{2–4}, all of which contain tetrahedrally coordinated metal atoms. In 1989, a family of microporous titanosilicates (generically denoted ETS) was discovered in which the metal atoms (Ti^{4+}) are octahedrally coordinated^{5–8}. A full understanding of the potential of any microporous solid to act as a molecular sieve and selective catalyst, and of the nature of the catalytic centres, requires that its structure be known. But that of the ETS materials has proved elusive because of the considerable degree of disorder that they contain. Using a combination of high-resolution electron microscopy, electron and powder X-ray diffraction, solid-state NMR, molecular modelling and chemical analysis, we have now been able to solve the structure of a prominent member of this family, ETS-10. This structure comprises corner-sharing SiO_4 tetrahedra and TiO_6 octahedra linked through bridging oxygen atoms. The pore system contains 12-membered rings and displays a considerable degree of disorder. Many ordered variants of ETS-10 exist, some of which are chiral.

In 1983 an important metal-substituted silicate, TS-1, (ref. 9) was discovered, it is a titanium-doped form of purely siliceous silicalite. The Ti (iv) adopts tetrahedral coordination, and these materials are highly active as oxidation catalysts. Recently^{5–8} an important new class of materials containing both octahedral and tetrahedral framework atoms has been discovered. Two titanosilicate members of this family are ETS-4 and ETS-10 (Engelhard Corporation titanosilicate) which shows adsorption

characteristics of microporous materials and, in the case of ETS-10, display characteristics indicating a wide-pore material.

It is difficult to determine the structure of ETS-10 because (1) it can only be synthesized as a powder with particle size $\sim 5 \mu\text{m}$, and (2) it exhibits a high degree of disorder—exemplified by broad powder X-ray diffraction reflections. A similar situation exists for zeolite- β , a wide-pore zeolite which was first synthesized in 1967 (ref. 10) but the structure of which was only determined in 1988 (ref. 11).

The strategy adopted for structural elucidation of ETS-10 was (1) examine the framework ring connectivity and local disorder with high-resolution electron microscopy (HREM), (2) determine the atomic make-up of the material by chemical analysis, (3) determine the local environment of silicon species by using ^{29}Si solid-state NMR, (4) refine a trial structure using distance least squares (DLS) analysis, (5) use this refined structure to stimulate the HREM images and (6) combine the minimized structure and the known disorder to stimulate both the powder X-ray diffraction and electron diffraction data.

ETS-10 was synthesized as follows⁵. A solution was made using 10.96 g sodium silicate solution (Na_2O 12 wt%, SiO_2 30 wt%), 1.0 g sodium hydroxide, 1.72 g potassium fluoride, 6.03 g titanium trichloride, 0.96 g sodium chloride and 10 g H_2O . Seed crystals (0.1 g) were added to the resulting gel and the crystallization performed in an autoclave under autogenous pressure at 200°C for 64 h. The crystalline product was filtered, washed and dried at room temperature. The X-ray diffraction pattern of the product shown in Fig. 1a has similar d -spacings to those reported in tabular form previously^{5,6}. Scanning electron microscopy showed that the external morphology of the crystals was compatible with tetragonal symmetry, with a particle size of $\sim 5 \mu\text{m}$.

Figure 2a shows the HREM image taken along one zone axis of the crystal and displays a tremendous amount of structural

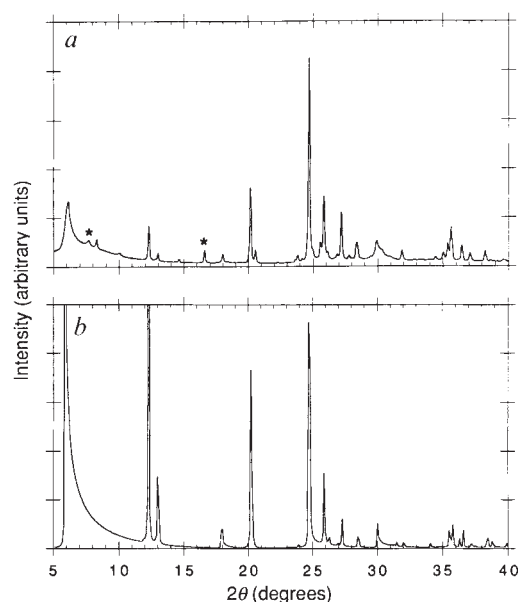


FIG. 1 X-ray diffraction pattern of ETS-10 (a) obtained using $\text{CuK}\alpha$ radiation. Both broad and narrow reflections are present, consistent with a highly crystalline but disordered material. The asterisks denote peaks due to very small amounts of ETS-4 impurity. The diffraction pattern was simulated (b) using the refined structure, including known disorder, using the DIFFAX routine¹²; all the important features can be simulated. The lower intensity of the experimental data at low 2θ is due to occluded water. Also, potassium and sodium cations were not included in the calculation. For such disordered materials a Rietveld refinement is not possible and so exact atomic coordinates cannot be determined from X-ray diffraction analysis. The framework connectivity, however, is verified.

TABLE 1 Atomic coordinates of polymorph B of ETS-10

Atom	Wyckoff notation	x	y	z
Ti1	4d	0.25000	0.75000	0.00000
Ti2	4a	0.00000	0.00000	0.00000
Ti3	8f	0.12756	0.87519	0.00888
Si4	4e	0.00000	0.81382	0.25000
Si5	8f	0.26308	0.96399	0.11336
Si6	8f	0.11514	0.10781	0.10674
Si7	8f	0.06118	0.76465	0.10474
Si8	8f	0.16265	0.66144	0.09800
Si9	8f	0.18935	0.98647	0.90805
Si10	8f	0.08492	0.08926	0.90112
Si11	8f	0.98990	0.79007	0.89975
Si12	8f	0.13588	0.63972	0.89331
Si13	8f	0.25024	0.93874	0.75973
Si14	4e	0.00000	0.69219	0.75000
O15	8f	0.27799	0.01448	0.19810
O16	8f	0.19964	0.91944	0.10436
O17	8f	0.24491	0.99903	0.01237
O18	8f	0.32945	0.92133	0.13300
O19	8f	0.18876	0.10200	0.18868
O20	8f	0.07117	0.04460	0.09640
O21	8f	0.12124	0.12028	0.00466
O22	8f	0.10563	0.82521	0.10037
O23	8f	0.05184	0.92412	0.01129
O24	8f	0.20104	0.82652	-0.00171
O25	8f	0.22194	0.70648	0.09161
O26	8f	0.10724	0.70233	0.12273
O27	8f	0.12815	0.62939	0.99483
O28	8f	-0.05908	0.85719	0.17945
O29	8f	0.03073	0.77029	0.18802
O30	8f	0.14891	0.92378	0.91557
O31	8f	0.05597	0.83159	-0.08833
O32	8f	0.02365	0.04687	0.90715
O33	8f	0.18365	0.70037	0.90441
O34	8f	0.13795	0.04504	0.87631
O35	8f	0.22549	0.97940	0.83124
O36	8f	0.00122	0.75968	0.00232
O37	8f	0.92387	0.83432	0.86693
O38	8f	0.97775	0.73550	0.82130
O39	8f	0.06271	0.64872	0.81130
O40	8f	0.30941	0.89186	0.82141

Polymorph B of ETS-10 has spacegroup $C2/c$, and unit cell parameters $a = b = 21.001 \text{ \AA}$; $c = 14.51 \text{ \AA}$; $\alpha = \gamma = 90^\circ$, $\beta = 111.12^\circ$

detail—even revealing the presence of three-ring units. The corresponding electron diffraction pattern is shown in Fig. 2b. Chemical analysis of the ETS-10 material showed that the Si/Ti ratio was, within experimental error, $\sim 5:1$. The ^{29}Si magic-angle spinning (MAS) NMR spectrum of ETS-10 is shown in Fig. 3.

Using the constraints imposed by the chemical analysis, the silicon connectivity from NMR and the fact that HREM showed the projected structure along two orthogonal axes to be identical, a trial structure was proposed which fitted the ring structure suggested by the HREM image. This structure is shown schematically in Fig. 4a. In order to validate this structure, approximate atomic coordinates were obtained from the model, then the coordinates of titanium, oxygen and silicon were optimized by DLS method. (For the DLS refinement the average bond lengths $\langle\text{Ti}-\text{O}\rangle$ and $\langle\text{Si}-\text{O}\rangle$ were taken as 1.94 and 1.625 $\text{\AA}$ respectively, and the average $\langle\text{Si}-\text{O}-\text{Si}\rangle$ and $\langle\text{O}-\text{Si}-\text{O}\rangle$ bond angles were taken as 145.0° and 109.47° respectively. Bond angles were given a weighting of 10% with respect to bond distances. After refinement the unit cell was adjusted by $\sim 2\%$ to fit parameters found from X-ray diffraction.) Using this structure, a through focus set of electron micrographs were simulated and the solution is shown as an inset in Fig. 2a. There is an excellent correlation between experimental and simulated images which is strong evi-

dence that the trial structure is correct regarding the framework connectivity.

The electron micrographs display the local structure of the crystal; in contrast, both the electron diffraction and the X-ray diffraction patterns indicate the crystal structure including disorder. Therefore, in order to simulate either diffraction pattern it is necessary first to understand the nature of the disorder in detail. The ETS-10 framework can be decomposed into sheets running parallel to the main orthogonal channels (say the a - b plane) which contain two orthogonal sets of Ti-O-Ti chains with associated silicate units. The disorder is manifested as a displacement of adjacent sheets in the a - b plane. This displacement is always $1/4$ unit cell in the a -direction and $1/4$ unit cell in the b -direction (Fig. 4B). Knowing these rules for the disorder,

and assuming a random stacking of sheets (that is, equal probability that adjacent sheets adopt each of the four possibilities), the electron or X-ray diffraction patterns can be calculated using the DIFFaX routine¹². These simulations are shown in Fig. 2c and Fig. 1b respectively and display excellent agreement between experiment and theory. On this basis the validity of the structural model is verified.

All possible ordered and disordered polytypes of ETS-10, and the nature of the line defects, can be explained in terms of different stacking sequences of the eight possible sheets. Two specific interesting examples of ordered polytypes are as follows: (1) where the stacking sequence is 1, 3, 6, 8 repeat. This will give a diagonal arrangement of the 12-ring pores—termed polymorph B; (2) where the stacking sequence is 1, 3, 1, 3, ... and so on.

FIG. 2 High-resolution electron micrograph of ETS-10 (a) recorded on a JEOL 400 kV electron microscope. Several features are immediately apparent from this image:

(1) ETS-10 contains large pores as previously reported^{5,6}; (2) the stacking disorder of these pores is immediately apparent; this disorder gives rise to the streaking in the electron diffraction pattern (b); (3) a host of planar defects are apparent where two pores appear to coalesce to give a large micropore. The density of these planar defects is $\sim 3\%$ and is a result of the handedness of a layer changing within the layer; (4) a considerable amount of fine structure is apparent which, particularly in the thinner parts of the crystal, allows the direct observation of three-ring units. The beam stability of these materials is excellent (compared to that of zeolites) making possible imaging with such high resolution. The high-resolution image was simulated (inset in a) using the optimized structure with a defocus of -450 Å and a crystal thickness of 60 Å. The electron-diffraction image was simulated (c) using the DIFFaX routine¹² by taking into account the known stacking disorder in ETS-10.

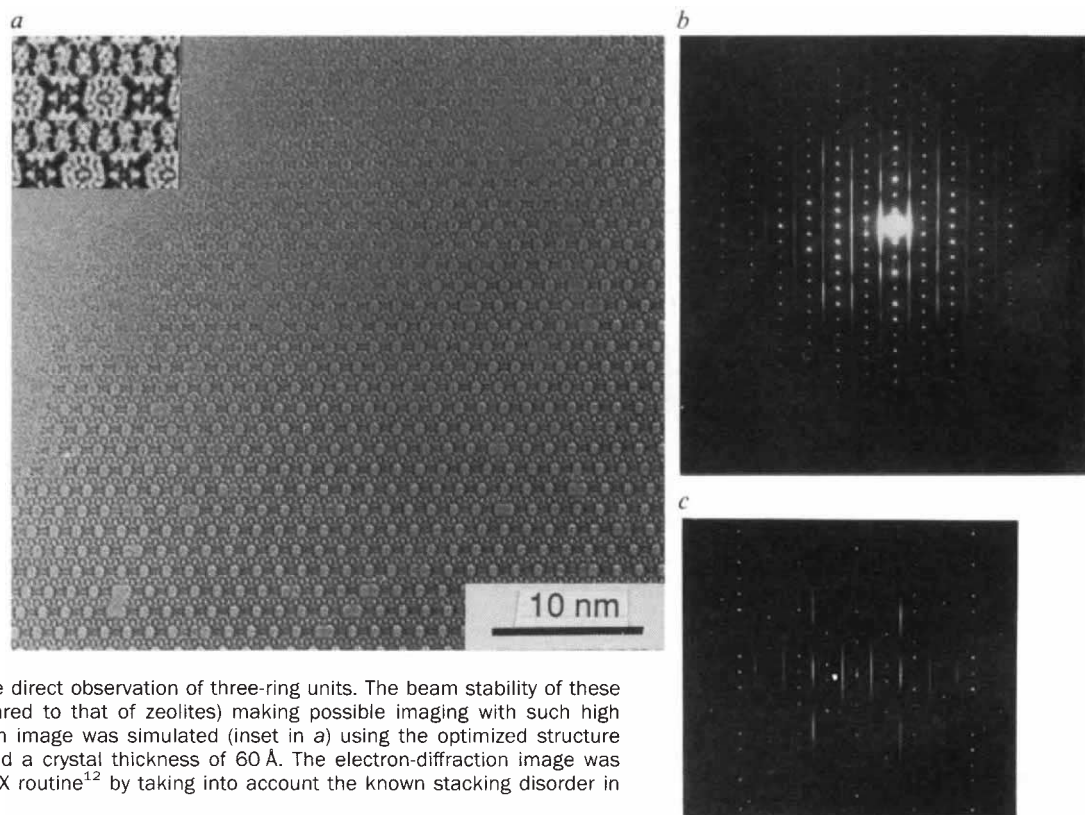
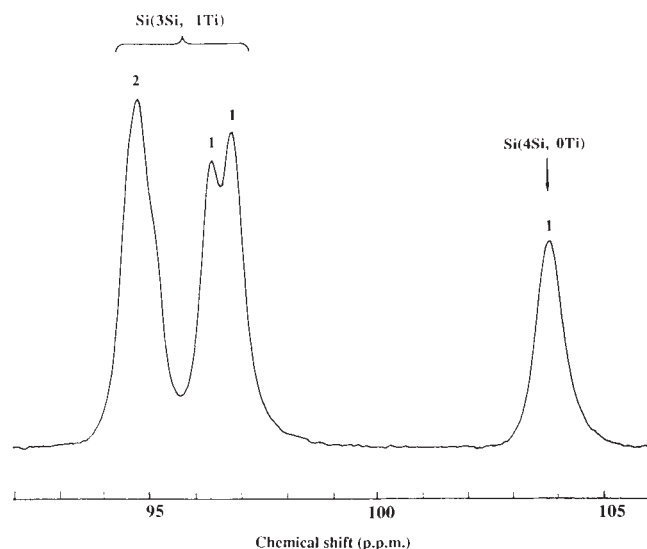


FIG. 3 The ETS-10 ^{29}Si solid-state NMR spectrum with magic-angle spinning (MAS) recorded on a Bruker 400MSL spectrometer. It shows four lines (one with a shoulder) with chemical shifts -94.1 , -95.8 , -96.5 and -103.3 p.p.m. referenced to tetramethylsilane. The intensity ratio of these resonances is $2:1:1:1$. By comparing the chemical shifts with other titanosilicate minerals such as zorite and lorenzenite (M. W. Anderson and A. Philippou, unpublished observations), the three resonances at -94.1 , -95.8 and -96.5 p.p.m. can all be assigned to $\text{Si}(3\text{Si}, 1\text{Ti})$ (that is, silicon connected through oxygen bridges to three silicon atoms and one titanium atom) and the resonance at -103.3 p.p.m. can be assigned to $\text{Si}(4\text{Si}, 0\text{Ti})$. These assignments are consistent with the framework connectivity of ETS-10 shown in Fig. 4. For the perfect $\text{C}2/c$ structure there are eight crystallographic types of $\text{Si}(3\text{Si}, 1\text{Ti})$. From an NMR point of view (that is, short-range order) these can be reduced to four distinct types in a $1:1:1:1$ ratio. Each silicon is either located in a 12-ring or a 7-ring, denoted $\text{Si}12$ or $\text{Si}7$ respectively. These silicon atoms are connected by way of an oxygen, to one Ti, one $\text{Si}(4\text{Si}, 0\text{Ti})$ and $2\text{Si}(3\text{Si}, 1\text{Ti})$. These latter two adjacent atoms are either $\text{Si}12$ or $\text{Si}7$ sites. The four possible $\text{Si}(3\text{Si}, 1\text{Ti})$ sites then become $\text{Si}12(12, 12)$, $\text{Si}12(12, 7)$, $\text{Si}7(7, 12)$ and $\text{Si}7(7, 7)$ —where the numbers in brackets refer to the ring type of the adjacent $\text{Si}(3\text{Si}, 1\text{Ti})$ site. These sites are labelled A, B, C and D respectively, in Fig. 4A. The ^{29}Si NMR spectrum is able to resolve three crystallographic types of $\text{Si}(3\text{Si}, 1\text{Ti})$ with two sites overlapping.



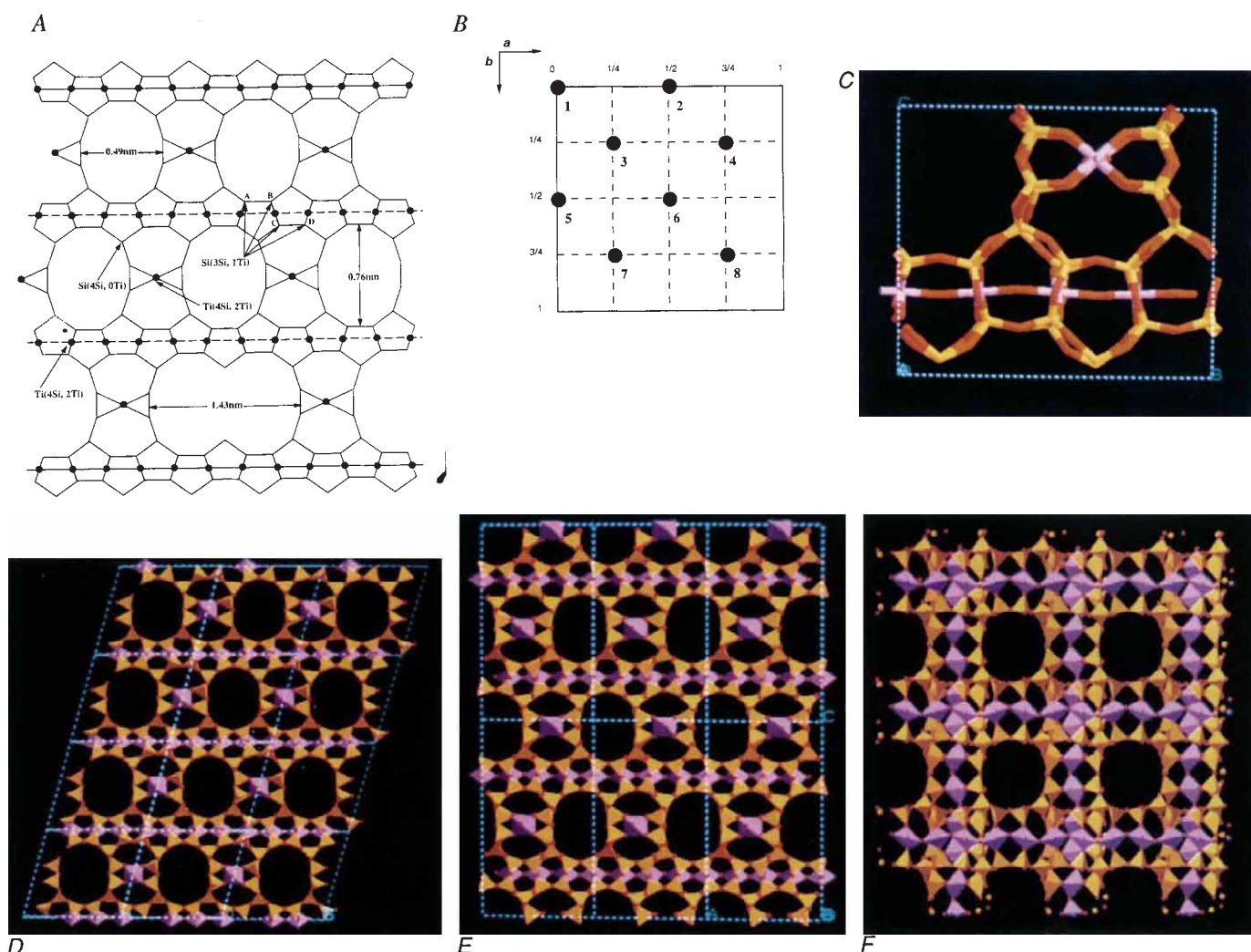


FIG. 4 The stoichiometry of this framework (A) is $\text{Si}_5\text{TiO}_{13}^{2-}$ which agrees with the chemical analysis, and the connectivity is exactly that suggested by ^{29}Si MAS NMR. The structure of ETS-10 is built up from sheets displaced according to the diagram (B). The dots represent the eight possible sheet displacements as a fraction of the unit cell. Two sheets, however, can only be connected if they are adjacent in this diagram; for example, $1 \rightarrow 3$ is allowed but $1 \rightarrow 2$ is not allowed. The basic building block for the disordered structure is most conveniently taken as a $\text{Si}_{40}\text{Ti}_8\text{O}_{104}^{16-}$ unit (C). The framework charge is balanced by either sodium or potassium cations. The titanium atoms are octahedrally coordinated and are linked to one another in a straight chain. These chains are aligned parallel to the main orthogonal channels alternating in direction as they are stacked along the c -axis. The titanium octahedra are surrounded by 4Si tetrahedra (each $\text{Si}(3\text{Si}, 1\text{Ti})$) connected in such a way as to produce two three-rings. Four distinct types of $\text{Si}(3\text{Si}, 1\text{Ti})$ are labelled A, B, C and D in the A, of which three can be resolved by NMR. These Si atoms are themselves connected together in purely siliceous

five-rings where the apical Si of each five-ring is the remaining $\text{Si}(4\text{Si}, 0\text{Ti})$. Rods consisting of a chain of titanium octahedra surrounded on both sides by silicon five-rings are joined together in a perpendicular arrangement to generate seven-rings and the complete stacking of these rods encompasses the large 12-rings ($7.6 \text{ \AA} \times 4.9 \text{ \AA}$). The projection down the $[110]$ zone axis is shown (D) for polymorph B (space group $C2/c$), and the projection down the $[100]$ zone axis is shown (E) for polymorph A (space group $P4_1$). Down the c zone axis, owing to the stacking disorder of the plane parallel to the c -axis, there are no simple pores, but a tortuous access may be gained by way of twelve-rings (F)—the structure has been cut away to show the window and does not represent a projection down the c -axis. The planar defects creating the larger pores can be explained by two sheets displaced by $1/2$ a unit cell. This produces a partial dislocation line and the resulting large micropores have a size $\sim 14.3 \text{ \AA} \times 7.6 \text{ \AA}$. Such dislocations may change the stoichiometry of the crystals slightly.

This will result in the 12-ring pores having a zig-zag arrangement—termed polymorph A. Polymorph B belongs to space group $C2/c$ with unit cell parameters $a = 21.00 \text{ \AA}$, $b = 21.00 \text{ \AA}$, $c = 14.51 \text{ \AA}$, $\alpha = 90.0^\circ$, $\beta = 111.12^\circ$ and $\gamma = 90.0^\circ$ (the atomic coordinates are given in Table 1). Polymorph A has either $P4_1$

or $P4_3$ symmetry with $a = 14.58 \text{ \AA}$, $b = 14.85 \text{ \AA}$, $c = 27.08 \text{ \AA}$, $\alpha = 90^\circ$, $\beta = 90^\circ$ and $\gamma = 90^\circ$. Consequently, polymorph A has a screw axis and therefore will display chiral symmetry. In fact there is a spiral channel along the c -direction, with a pitch of $27.08 \text{ \AA}$. $\square$

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Steep declines in atmospheric base cations in regions of Europe and North America

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HUMAN activities have caused marked changes in atmospheric chemistry over large regions of Europe and North America. Although considerable attention has been paid to the effects of changes in the deposition of acid anions (such as sulphate and nitrate) on terrestrial and aquatic ecosystems^{1–7}, little is known about whether the concentrations of basic components of the atmosphere have changed over time^{8,9} and what the biogeochemical consequences of such potential changes might be. In particular, there has been some controversy^{8–12} as to whether declines in base-cation deposition have countered effects of recent reductions in SO₂ emission. Here we report evidence for steep declines in the atmospheric concentrations of base cations (sum of non-sea-salt Ca²⁺, Mg²⁺, K⁺ and Na⁺) over the past 10 to 26 years from high-quality precipitation chemistry records in Europe and North America. To varying but generally significant degrees, these base-cation trends have offset recent reductions in sulphate deposition in the regions examined. The observed trends seem to be ecologically important on decadal timescales, and support earlier contentions^{8–10} that declines in the deposition of base cations may have contributed to increased sensitivity of poorly buffered ecosystems.

We selected records from high-quality precipitation monitoring stations in North America and Europe with ≥ 10 yr of continuous data, and where collection and analytical methods have been kept consistent over time (Fig. 1). We modelled trends in annual volume-weighted mean (VWM) base-cation concentrations and deposition both as simple linear and as quadratic regressions (Table 1). Due to strong influence from marine air trajectories, we subtracted the contributions of sea-salt aerosols¹³ to precipitation chemistry at all European monitoring sites^{14,15} and at North American sites located <100 km from the coast (Fig. 1). The North American data were derived from the Hubbard Brook Experimental Forest (HBEF)¹⁶, the National Atmospheric Deposition Program/National Trends Network

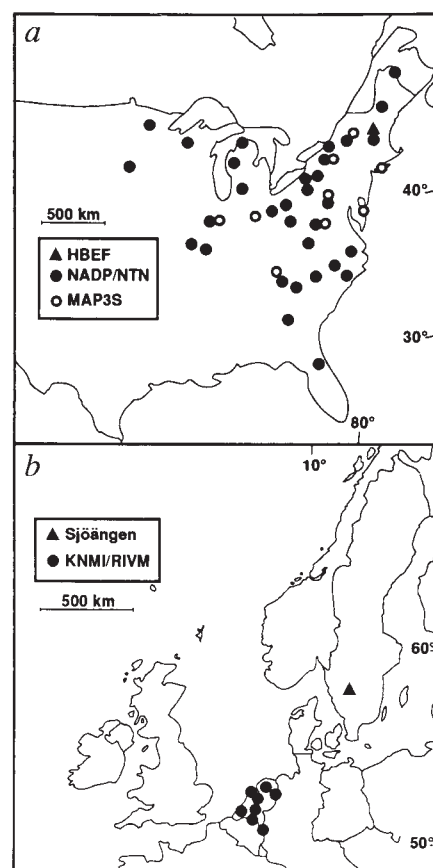


FIG. 1 Locations of precipitation monitoring stations. a, Eastern United States: HBEF (▲); NADP/NTN network (●); MAP3S network (○). b, Europe: KNMI/RIVM network (●); Sjöängen (▲).

METHODS. Measures of base cations in precipitation are especially sensitive to contamination by local sources of dust and to changes in collection or analytical methods. The HBEF record consists of weekly bulk precipitation samples from collectors in watershed no. 6 (ref. 16) during calendar years with complete anion and cation chemistry (1965–90). NADP/NTN stations collect weekly wet-only precipitation samples throughout the United States¹⁷. MAP3S stations collect event-based wet-only precipitation samples¹⁸. KNMI/RIVM stations are located away from immediate industrial emission areas, collecting monthly bulk precipitation samples^{5,14}. Long-term and seasonal trends were generally similar between the eight stations¹⁴. The Sjöängen record consists of monthly samples, and has been carefully evaluated for effects due to local contamination and changes in methods. A comparison against 12 satellite stations during 1973–75, installed in an <100 km diameter area, showed Sjöängen to be regionally representative. Base-cation concentrations at Sjöängen followed closely arithmetic mean concentrations from the satellite stations over a 15-month period (Pearson's $r = 0.95$; $P < 0.001$), indicating negligible local effects. A 45-month side-by-side comparison of bulk and wet-only collectors showed that seasonal trends in base cations and SO₄²⁻ were not statistically different between collector type. On average, annual VWM concentrations of base cations were 3.4 μEq l⁻¹ higher for the bulk collector, probably because of dry deposition. Long-term wind records were evaluated at representative weather stations: Concord airport (43° 12' N, 71° 30' E, ~100 km from HBEF), New Hampshire (1965–89); Sätenäs airport (58° N, 12° E, ~100 km from Sjöängen) in southern Sweden (1971–92); Rotterdam airport (51° 57' N, 4° 27' E); Leeuwarden (53° 13' N, 5° 46' E) in the Netherlands (1971–90). (Both Rotterdam airport and Leeuwarden are stations in our composite record.)

(NADP/NTN)¹⁷ and the MAP3S network¹⁸ (Fig. 1a). The HBEF record (bulk collectors) is the longest existing high-quality record of precipitation chemistry in North America¹. We confined our analyses of the NADP/NTN network (wet-only collectors) to 32 eastern US stations, 20 with records between

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TABLE 1 Long-term trends in base cations and sulphate in precipitation

Site	Period	Precip. volume (mm yr ⁻¹)	Base cations conc. trend <i>b</i> , (s.e.) (μEq l ⁻¹ yr ⁻¹)	<i>r</i> ²	Base cations per cent change	Sulphate conc. trend <i>b</i> , (s.e.) (μEq l ⁻¹ yr ⁻¹)	Sulphate per cent change	<i>r</i> ²	Sulphate trend balanced by base cations (%)	Base cations depos. trend <i>b</i> , (s.e.) (Eq ha ⁻¹ yr ⁻¹)
The Netherlands										
(1) Composite station	1978–87	811	-1.5(0.39)*†	0.67	-32	-4.4(0.81)‡	-29	0.79	34	-15.1(2.9)‡
(2) Lelystad	1978–89	805	-1.7(0.32)†‡	0.75	-47	-3.2(0.85)*§	-29	0.60	53	-17.7(2.8)‡
(3) Witteveen	1978–89	816	-0.9(0.36)†‡	0.41	-39	-3.2(0.89)*	-30	0.56	28	-10.7(2.4)‡
Sweden										
(1) Sjöängen, bulk	1971–81	568	-1.3(0.47)	0.46	-54	-1.9(0.74)	-25	0.43	68	-7.0(3.0)
(2) Sjöängen, wet-only	1977–89	578	-0.8(0.16)†	0.68	-62	-1.2(0.31)*	-22	0.59	63	-2.9(1.1)
(3) Composite station	1971–89	591	-0.8(0.17)†	0.53	-74	-1.4(0.26)‡	-34	0.63	54	-3.8(1.1)*
North America										
(1) Hubbard Brook	1965–89	1414	-0.4(0.06)†*	0.70	-49	-1.1(0.11)‡	-43	0.80	38	-5.1(0.92)‡
(2) NADP, means of regression slopes										
Eastern US (32 stns)	1979–90	NA	-1.0(0.07)‡	NA	NA	-1.0(0.10)‡	NA	NA	95	-8.3(0.9)‡
Midwest US (12 stns)	1979–90	NA	-1.2(0.15)‡	NA	NA	-1.3(0.12)‡	NA	NA	92	-9.4(1.7)‡
Northeast US (12 stns)	1979–90	NA	-0.9(0.06)‡	NA	NA	-1.1(0.17)‡	NA	NA	84	-7.7(1.2)‡
Southeast US (8 stns)	1979–90	NA	-0.7(0.12)‡	NA	NA	-0.6(0.20)	NA	NA	110	-7.4(1.7)‡
(3) MAP3S										
Northeast US (9 stns)	1978–88	NA	NS	NA	NA	-0.8(0.20)*	NA	NA	NA	NA

Summary statistics of long-term trends in annual volume-weighted means of base cations and sulphate in precipitation at stations in Europe and North America. Linear regressions are of the form $Y = bX + a$, where b and a are coefficients, Y is ion concentration or deposition and X is year. Quadratic regressions are of the form $Y = b_1X + b_2Z^2 + a$, where b_1 equals the linear regression slope (b , above), b_2 and a are coefficients, Z^2 is a quadratic term corrected by subtraction of $\bar{X}$ ($Z = (X - \bar{X})$). Regression residuals were inspected. Only significant ($P < 0.05$) terms were included in the final model. The quadratic term was only significant for base cations at HBEF. The addition of an autoregressive term with a lag time of 1–5 yr had little effect on standard errors and significance levels (s.e., standard error; NA, information not applicable; NS, not significant at $P < 0.05$).

* Significance level < 0.01 .

† To allow conservative interpretation of trends, we excluded 1978 values which were somewhat elevated. Slopes are stronger with 1978 values included: composite station = $-2.5(0.65)^*$; Lelystad = $-2.2(0.41)^{†‡}$; and Witteveen = $-1.5(0.45)^*$.

‡ Significance level < 0.001 .

§ We excluded the slightly elevated 1982 data. As 1982 occurs in the middle of the record, exclusion had little effect on slope but a strong effect on the error estimate and significance of trend. Values with 1982 included were: $-3.6(1.4)^{†‡}$.

| Significance level < 0.05 .

¶ Quadratic coefficient significant at $P < 0.01$.

1979 and 1990 and 12 with records between 1980 and 1990. We used data for 1978–88 from all nine stations of the MAP3S network (wet-only collectors). In Europe we evaluated data from national networks in the Netherlands and Sweden (Fig. 1b). Three records were selected from the KNMI/RIVM network^{5,14} in the Netherlands (bulk collectors): (1) a composite record based on arithmetic averages of annual VWM concentrations from eight stations (1978–87)¹⁴, (2) data from Lelystad (1978–89), and (3) data from Witteveen (1978–89). Lelystad and Witteveen are the longest existing records with consistent sampling methodology within the network. Base-cation trends before ~1975 were compromised at most Swedish network stations by dust contamination associated with poor location (evaluated against new stations) and the design of a certain collector model ("Egner skåp"). The longest high-quality, regionally representative (Fig. 1) Swedish record comes from Sjöängen¹⁵ (Fig. 1b), consisting of 11 years of bulk and 13 years of wet-only data during 1971–89. We selected three records: (1) bulk precipitation (1971–81), (2) wet-only precipitation (1977–89), and (3) composite averages from both collector types (1971–89). The composite record was corrected for average between-collector difference in annual VWM base-cation concentrations ($3.4 \mu\text{Eq l}^{-1}$), estimated during a 45-month side-by-side calibration of collector types.

Our results show strong and statistically significant declines in concentrations and deposition of base cations in both North America and Europe (Table 1 and Fig. 2). The strongest rates of concentration decline (range: -1.7 to $-0.9 \mu\text{Eq l}^{-1} \text{yr}^{-1}$) occurred in the Netherlands during 1978–87 (Fig. 2a). Declines were similarly strong in Sweden (Fig. 2b) with linear regression slopes ranging from $-1.3 \mu\text{Eq l}^{-1} \text{yr}^{-1}$ for the bulk precipitation record (1971–81) to $-0.8 \mu\text{Eq l}^{-1} \text{yr}^{-1}$ for the wet-only record (1977–89). A slightly weaker ($-0.4 \mu\text{Eq l}^{-1} \text{yr}^{-1}$), but highly significant ($P < 0.001$; $r^2 = 0.7$), base-cation decrease occurred during 26 years (1965–90) at the HBEF in the northeastern United States (Fig. 2c). The HBEF regression trend had a significant

($P < 0.01$) quadratic term (Table 1) indicating a more rapid base-cation decrease in the 1960s and early 1970s, and only relatively small changes after that period (Fig. 2c). Trends in the Netherlands may also have levelled off during later years, as suggested by our data (Fig. 2) and by the lack of significant trends in base cations during 1985–90 at Arnhem¹⁹. In contrast, data from 30 additional stations (15 containing either single wet-only collectors or both wet-only and bulk collectors) distributed throughout Sweden showed continued linear declines during 1983–91 for both excess Ca^{2+} ($-7\% \text{yr}^{-1}$; $P < 0.01$) and Mg^{2+} ($-9\% \text{yr}^{-1}$; $P < 0.02$), but not excess K^+ ($P > 0.48$) or Na^+ ($P > 0.97$).

Over shorter periods we may approximate observed changes by linear regression trends. Linear trends for base-cation concentrations during 1979–90 were negative at all 32 NADP/NTN eastern US stations (Fig. 3a). The distribution of regression slopes was significantly different from zero ($P < 0.001$; t -test), with a mean base-cation decline of $-1.0 \mu\text{Eq l}^{-1} \text{yr}^{-1}$ (range: -0.3 to $-2.4 \mu\text{Eq l}^{-1} \text{yr}^{-1}$) (Fig. 3). Rates of decline varied geographically ($P < 0.03$; analysis of variance) with highest average values in the midwestern ($-1.2 \mu\text{Eq l}^{-1} \text{yr}^{-1}$; $n = 12$; n , number of sites), intermediate in the northeastern ($-0.9 \mu\text{Eq l}^{-1} \text{yr}^{-1}$; $n = 12$) and lowest in the southeastern United States ($-0.7 \mu\text{Eq l}^{-1} \text{yr}^{-1}$; $n = 8$). The rank reflects regional-scale differences in base-cation concentrations, which are higher in the midwestern, intermediate in the northeastern and least in the southeastern United States²⁰. Further support for these regional-scale patterns comes from a semivariance analysis which indicated strong variation in the decline rates at regional geographical scales ($> 1,200 \text{ km}$ distance) and low to non-existent spatial autocorrelation over more local scales ($< 1,200 \text{ km}$).

Although regression slopes for base cations were slightly negative at five out of nine MAP3S stations, the overall distribution of slopes was not significantly different from zero ($P > 0.2$; t -test) nor were any trends at individual sites statistically significant. However, four out of nine MAP3S sites showed statistically sig-

nificant ($P < 0.05$) declines in H^+ concentration over the same period. This apparent difference in base-cation trends between the NADP/NTN and the MAP3S networks may be related to differences in sampling methodology (weekly compared to event-based samples), sampling periods (1979–90 compared to 1978–88) or numbers of monitoring stations (32 compared to 9) between the two networks. A similar treatment of data from HBEF during 1978–88 also fails to give a statistically significant base-cation trend ($P > 0.17$) which either indicates that the decrease had levelled off, as indicated by Fig. 2c, or that the sampling period (11 yr) together with year-to-year variations do not permit detection of underlying trends.

The observed trends (Table 1) translate into substantial long-term changes in precipitation chemistry. For example, concentrations of non-sea-salt base cations declined by 32% over 10 yr in the Netherlands (composite record), by 74% over 19 yr in Sweden (composite record), and by 49% over 26 yr at HBEF, North America (Table 1). Declines were generally dominated by Ca^{2+} which contributed, on the basis of charge equivalents, ~81% to the total base-cation decline in the Netherlands, 65% in Sweden and 48% at HBEF.

The base-cation declines were quantitatively significant relative to recent declines in precipitation SO_4^{2-} in Europe^{4,6}, and North America^{1,3} (Table 1). On the basis of charge equivalents, base cations balanced 34% of the concurrent SO_4^{2-} decline in the Netherlands (composite record) (Fig. 2a), 54–68% in the Swedish records (Fig. 2b), and 38% in the HBEF record (Fig. 2c) (Table 1). The average rate of base-cation decrease ($-1.0 \mu\text{Eq l}^{-1} \text{yr}^{-1}$) for the eastern US NADP/NTN network

did not differ from the average rate of decrease in SO_4^{2-} ($-1.0 \mu\text{Eq l}^{-1} \text{yr}^{-1}$) (Table 1; Fig. 3a compared to Fig. 3b). These results, with the exception of the nine MAP3S stations (Table 1), indicate that recent SO_2 emissions reductions in Europe^{4,6} and North America^{1,3} have not been accompanied by equivalent declines in net acidity related to sulphate in precipitation. Instead, SO_4^{2-} declines have to varying degrees been offset by simultaneous declines in base cations.

Our results support recent contentions^{8,10} that declines in atmospheric base cations may be of ecological significance. The depletion of exchangeable base cations in sensitive soils is currently thought to represent a major detrimental effect of acid deposition on terrestrial ecosystems^{6,10,21} and may contribute to forest decline^{22,24}. Deposition of SO_4^{2-} in the form of a salt (for example, $CaSO_4$), as opposed to the acid form (H_2SO_4), is not associated with such long-term acidification of soils due to base-cation loss from exchange complexes²⁵. Thus, our observation that SO_4^{2-} declines have to a significant degree been balanced by declines in base cations (as opposed to H^+ ; Table 1) indicates that recent emission reductions have not necessarily resulted in equivalent improvements in the base status of sensitive soils.

Our results indicate that atmospheric deposition, at least in the beginning of the period discussed here, can be a quantitatively important source of base cations to sensitive soils. Our highest regression estimates of bulk Ca^{2+} deposition for the Netherlands ($307 \text{ Eq ha}^{-1} \text{yr}^{-1}$), Sweden ($85 \text{ Eq ha}^{-1} \text{yr}^{-1}$) and HBEF ($124 \text{ Eq ha}^{-1} \text{yr}^{-1}$) are of the same order of magnitude as estimates of biomass demand in second-growth temperate forests of Europe and North America ($\sim 100\text{--}800 \text{ Eq ha}^{-1} \text{yr}^{-1}$)^{7,16,26}. Our input estimates are conservative as they do not include dry deposition, which may equal up to 30% of total deposition^{8,27}. Recent isotope studies in Europe and North America^{10,26} and cation budgets^{28,29} provide further evidence that atmospheric inputs may be quantitatively important as a source of base cations to vegetation in nutrient-poor conditions. Current exchangeable Ca^{2+} pools are as low as $\sim 2\text{--}8 \text{ kEq ha}^{-1}$ within the rooting zone ($\sim 0\text{--}35 \text{ cm}$ depth) of sensitive soils in Europe^{26,30} (B. Nihlgård, personal communication) and North America^{23,29}. Given a theoretical Ca^{2+} pool of 4 kEq ha^{-1} , our highest regression estimates of bulk Ca^{2+} deposition from HBEF ($124 \text{ Eq ha}^{-1} \text{yr}^{-1}$), Sweden ($85 \text{ Eq ha}^{-1} \text{yr}^{-1}$) and the Netherlands ($307 \text{ Eq ha}^{-1} \text{yr}^{-1}$) could, respectively, supply the equivalent of 62, 42 and 154% of the pool over a 20-yr period. In contrast, at current lower deposition, atmospheric inputs could supply only 25, 12 and 104% to these sites during a 20-yr period.

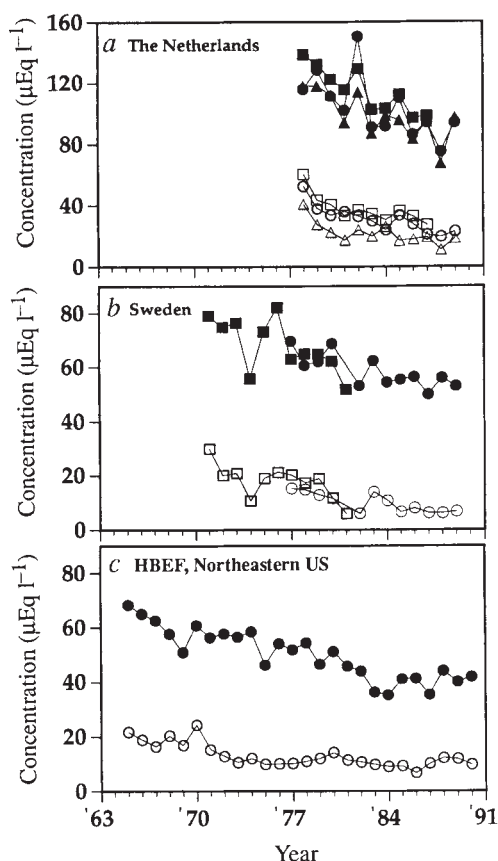


FIG. 2 Long-term trends in annual VWM concentrations of base cations (open symbols) and SO_4^{2-} (closed symbols) in precipitation. a, The Netherlands; composite record of eight monitoring stations ($\square$ and $\blacksquare$); Lelystad ($\circ$ and $\bullet$); Witteveen ($\triangle$ and $\blacktriangle$). b, Sjöäng, Sweden; bulk collector ($\square$ and $\blacksquare$); wet-only collector ($\circ$ and $\bullet$). c, HBEF, northeastern United States ($\circ$ and $\bullet$). (Horizontal axis shows 1963 to 1991.)

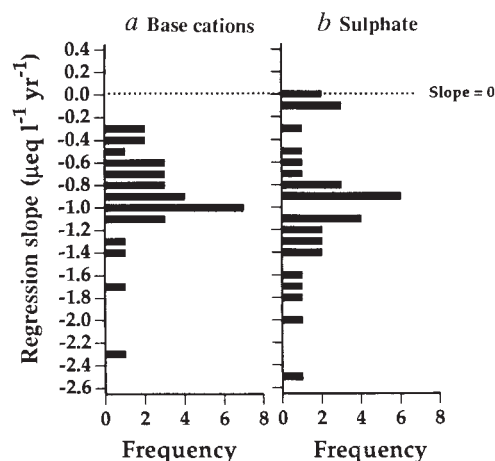


FIG. 3 Frequency distributions of linear-regression trends in annual VWM concentrations of base cations (a) and SO_4^{2-} (b) at 32 sites in the NADP/NTN network.

These calculations suggest that the observed atmospheric base-cation trends are ecologically relevant on the scale of decades for poorly buffered ecosystems.

Several factors may contribute to the observed declines in atmospheric base cations in Europe and North America. Sources of non-sea-salt base cations to the atmosphere include point-source emissions from fuel combustion and industrial processes, as well as open-source emissions associated with traffic on unpaved roads, agricultural tillage practices and natural particle emissions from forest fires or wind erosion of arid soils^{9,20,31–37}. Emissions from wind erosion are particularly sensitive to trends in velocity distributions of winds and in amounts of precipitation^{32–33}. As a first-order evaluation of these factors we examined long-term records from the HBEF, the Netherlands and Sjöängen, Sweden (Fig. 1). Strong wind events were defined as $>8 \text{ m s}^{-1}$ daily mean velocity at 10 m above ground in the Netherlands (10% of all days, 1971–90) and Sjöängen (7% of all days, 1971–93) and $>6 \text{ m s}^{-1}$ at 6 m above ground for HBEF (6% of all days, 1963–89). For all sites, we found no evidence of trends in amounts of precipitation or in number of days per year with strong winds, indicating that base-cation declines were not caused by secular changes in these factors. In contrast, regional point-source emissions of particulate matter have declined strongly in areas of Europe and North America. Locally-weighted regression fits to published emission data showed secular declines of 70% in the US (1960–91)³⁴, 24% in former East Germany (1975–90)³⁵, 75% in former West Germany (1966–90)³⁵, 51% in the UK (1970–90)³⁶, 63% in the Netherlands (Netherlands Central Bureau of Statistics, personal communication) (1970–90) and 82% in Sweden (1960–80)³⁷. Although poorly known, changes in open-source emissions (for example, unpaved roads³¹) may also have contributed to the decline in atmospheric deposition of base cations. □

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Role of the Bering Strait in controlling North Atlantic ocean circulation and climate

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RECENT climate records from ice cores and deep-sea sediments suggest that there has been considerable climate variability in the North Atlantic region over the past 250,000 years^{1–3}. Much of this variability may be linked to changes in thermohaline circulation in the North Atlantic ocean^{4–6}. Model studies^{7–9} have demonstrated that changes in the flux of fresh water to the ocean, resulting from changes in atmospheric transport or the waxing and waning of ice sheets, can have a significant effect on the thermohaline circulation. Here we present model simulations showing that increased flow of fresher North Pacific water through the Bering Strait into the northern North Atlantic can also affect the thermohaline circulation, by suppressing North Atlantic Deep Water formation; however, decreased flow does not necessarily cause deep-water formation to begin again. In our model, flow through the Bering Strait depends on eustatic sea level and the salinity difference between the North Atlantic and North Pacific oceans. We suggest that the higher sea level during the last interglacial period¹⁰, leading to greater flow through the Bering Strait, may have made the North Atlantic thermohaline circulation more sensitive than it is at present to fluctuations in the hydrological cycle, which may explain recent observations¹ indicating that climate variability was greater then than it is today.

The Bering Strait between Alaska and Siberia forms a short, but constricted, passage for flow between the North Pacific and North Atlantic oceans. At present, northward flow through this 85-km-wide and 50-m-deep strait carries fresh North Pacific surface water of ~ 32.5 parts per thousand (p.p.t.) salinity at a rate of almost 1 Sverdrup ($1 \text{ Sv} = 10^6 \text{ m}^3 \text{ s}^{-1}$) into the Arctic Sea¹¹. During glacial time with low sea level, this passage was closed. These properties of the strait and the flow through it have led to suggestions that the Bering Strait may influence climate^{12–14}. Although short-term rates of transport through the strait vary widely in response to changing winds, the standard deviation of annual transport estimates vary by only $\sim 0.1 \text{ Sv}$ (ref. 11). Mean, northward flow is probably driven by higher sea level (by $\sim 0.4 \text{ m}$) in the North Pacific. This sea level difference is due to lighter, fresher water in the upper 1,000 m of the North Pacific compared to the North Atlantic^{12,15}. An atmospheric transport of water vapour of $\sim 0.3 \text{ Sv}$ from east to west across Central America maintains this inter-ocean salinity difference¹⁶. Although turbulent friction may be expected to be important in the shallow Bering Strait, results of a numerical model¹⁷ (including friction and the Earth's rotation) suggest that mean flow

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through the strait, whose width is on the order of the barotropic Rossby radius of deformation, may be limited by geostrophic control such that

$$q_{BS} = g(\eta_{NP} - \eta_{NA})Hf^{-1} \quad (1)$$

where q_{BS} is the maximum, mean flow through the strait, g is the acceleration due to gravity, $(\eta_{NP} - \eta_{NA})$ is the sea level difference between the two oceans which limits the sea level difference across the strait, H is the water depth in the strait and f is the Coriolis parameter. For the simple model considered below, it is then reasonable to use a flow law based on equation (1) where $(\eta_{NP} - \eta_{NA})$ may be taken to be proportional to $(\rho_{NA} - \rho_{NP})$, the density difference between the two oceans.

We have formulated a simple, three-box model of ocean thermohaline circulation (Fig. 1) which extends earlier

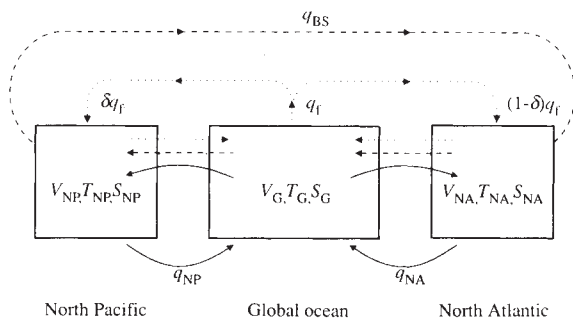


FIG. 1 The three-box model of ocean thermohaline circulation including the Bering Strait connection. NP and NA stand for the northern North Pacific and North Atlantic oceans respectively, and G stands for the rest of the global ocean. V , T and S are box volumes, temperatures and salinities. All are held fixed except S , which is a variable of the problem. $q_{NP,NA}$ is the 'deep' thermohaline flow, defined as positive from the NP, NA boxes to the G box. There is an equal and opposite return flow between respective boxes at the 'surface'. q_{BS} is the Bering Strait flow defined positive from the NP to the NA box. There is a compensation flow through the G box back to the box from which q_{BS} originates. q_f is the net fresh-water input (precipitation and runoff minus evaporation) to the NP and NA boxes associated with atmospheric transport of water vapour from the G box. δ is the fraction of q_f entering the NP box. Thus $(2\delta - 1)q_f$ is the excess of water vapour flux to the North Pacific over that to the North Atlantic. There is a compensating flow in the ocean back to the G box. Water density ρ follows a linear equation of state:

$$\rho/\rho_0 = 1 + \beta S - \alpha T \quad (2)$$

where ρ_0 is a reference density and β and α are the salinity and thermal expansion coefficients. Thermohaline flow follows

$$q_{NP,NA} = v' \rho_0^{-1} (\rho_{NP,NA} - \rho_G) = v' [\beta (S_{NP,NA} - S_G) - \alpha (T_{NP,NA} - T_G)] \quad (3)$$

where v' is a flow parameter. Flow in the model Bering Strait follows

$$q_{BS} = \gamma' \rho_0^{-1} (\rho_{NA} - \rho_{NP}) = \gamma' [\beta (S_{NA} - S_{NP}) - \alpha (T_{NA} - T_{NP})] \quad (4)$$

where γ' is another flow parameter. Equation (4) derives from equation (1) and the discussion in the text. It follows likewise that γ' varies linearly with H , the water depth in the Bering Strait. We define non-dimensional flow parameters $(v, \gamma) = (v', \gamma'/10^9 \text{ m}^3 \text{ s}^{-1})$. Salt conservation demands

$$V_{NP} \dot{S}_{NP} = -\delta q_f S_{NP} + |q_{NP}| (S_G - S_{NP}) + \begin{cases} q_{BS} (S_G - S_{NP}), & q_{BS} \geq 0 \\ q_{BS} (S_{NP} - S_{NA}), & q_{BS} < 0 \end{cases} \quad (5)$$

$$V_{NA} \dot{S}_{NA} = -(1-\delta) q_f S_{NA} + |q_{NA}| (S_G - S_{NA}) + \begin{cases} q_{BS} (S_{NP} - S_{NA}), & q_{BS} \geq 0 \\ q_{BS} (S_{NA} - S_G), & q_{BS} < 0 \end{cases} \quad (6)$$

$$S_G = (S_m V_m - S_{NP} V_{NP} - S_{NA} V_{NA}) V_G^{-1} \quad (7)$$

where S_m and V_m are ocean mean salinity and volume, and dots indicate time derivatives. Mass (volume) conservation holds for the formulae above. Steady-state solutions are found by setting the left-hand side of equations (5) and (6) to zero. Fresh-water fluxes associated with waxing and waning of ice sheets are not included as we deal here with steady-state solutions only.

work¹⁸⁻²⁰ to include a Bering Strait connection and asymmetric fresh-water forcing. Our model ocean is forced by prescribed temperatures and fresh water fluxes. Such a 'mixed boundary condition' problem, for which the competitive effects of heat and fresh-water forcing on water density are decoupled, permits multiple, stable, steady-state solutions for ocean thermohaline circulation given constant forcing¹⁸. We find up to four such modes, as in other such three-box models²⁰: the 'halocline catastrophe'⁶ (no deep-water formation in the North Atlantic or North Pacific, mode 1), the 'conveyor belt'²¹ (deep-water formation in the North Atlantic, mode 2, resembling the thermohaline circulation in the modern ocean), the 'reverse conveyor belt' (deep-water formation in the North Pacific, mode 3) and 'twin polar convection' (deep-water formation in the North Atlantic and North Pacific, mode 4). Switchovers between modes 1 and 2 may explain large climate variability in the North Atlantic region for timescales of hundreds to thousands of years, as implied by ice-core and deep-sea sediment data¹⁻³.

Both the 'halocline catastrophe' and the 'conveyor belt' modes are found to be possible stable, steady-state solutions when the Bering Strait is closed (Fig. 2, $\gamma = 0$, -50 m sea level). Assume that initially sea level is low and the 'conveyor belt' mode is on. Rising sea level opens the strait, and a flow develops through it from the North Pacific to the North Atlantic due to lower salinity, lower density and higher sea level in the former ocean. This flow freshens the North Atlantic surface layer and thereby weakens the thermohaline circulation in this ocean. As the sea level continues to rise, a point is reached (Fig. 2, $\gamma = 0.77$, +14 m sea level) beyond which the 'conveyor belt' mode collapses to give the 'halocline catastrophe' mode. On the way to this critical point, the North Atlantic-North Pacific salinity difference has decreased from 2.6 to 1.8 p.p.t., the Bering Strait flow has increased from 0 to 1.1 Sv, the main ocean (G box in Fig. 1)-North Atlantic salinity difference has increased from 0.9 to 1.6 p.p.t. and the North Atlantic thermohaline circulation has decreased from 15.3 to 10.0 Sv. Whereas switchovers between the two modes would lead to drastic changes in circulation (and, by inference, in heat transport towards the pole) in the North Atlantic, the circulation in the North Pacific is quite similar for both modes, with upwelling of between 4.5 and 5.5 Sv for the range of γ shown in Fig. 2. It should be noted that, in the model, falling sea level will not switch the 'conveyor belt' back on from the 'halocline catastrophe' (Fig. 2). Additional mechanisms would be required for this.

The Bering Strait flow is coupled to internal ocean dynamics and its addition to the basic thermohaline circulation model brings about qualitative changes in the behaviour of the system (Fig. 3). Asymmetry in fresh-water forcing promotes the 'conveyor belt' (mode 2), suppressing mode 3, its mirror image, whereas the Bering Strait flow promotes symmetric solutions (modes 1 and 4) while suppressing the asymmetric ones (modes 2 and 3) through negative salinity and density feedbacks. (In the limit of a deep, wide strait, North Pacific and North Atlantic waters would intermingle freely and the system would be reduced to one high- and one low-latitude box with only two possible (symmetric) stable, steady-state solutions for constant forcing¹⁸.) A rich solution structure results from the interplay between these tendencies. For instance, the possibility arises for deep-water formation in the North Pacific ocean during deglaciation as the Bering Strait opens to allow removal of fresh water from the North Pacific (point B in Fig. 3). We ran model simulations with q_{BS} as a free parameter in equations (5) and (6) (Fig. 1). As above, the 'conveyor belt' (mode 2) was suppressed as q_{BS} was increased; conversely increasing q_{BS} as a free parameter promotes mode 3 by advection of salt from the 'south' in the compensation flow.

These results are probably relevant to the problems of predicting future climate change and of understanding climate change in the past. If, as suggested above, the present ocean-atmosphere system lies near a critical point for the shutdown of

the 'conveyor belt' mode, continuing global warming and climate change due to the actions of Man may lead to this shutdown and subsequent cooling in the North Atlantic region. Although sea level changes of the order of 10 m are not likely in the near future²², global warming may lead to a stronger hydrological cycle²³ and an increase of q_f , the net atmospheric transport of water vapour toward the pole. This would favour the collapse of deep-water formation in the North Atlantic (Fig. 3). On the other hand, global warming may lead to an increase in the temperature difference between high and low latitudes in the ocean: whereas temperatures in the polar ocean are anchored by the freezing point of sea water, ocean temperature at low and mid latitudes may be expected to rise. This would favour the 'conveyor belt' mode (Fig. 1, equation (3)). If the present system is near the critical point, possible finite-amplitude perturbations, for instance a temporary increase in runoff from the Alaskan,

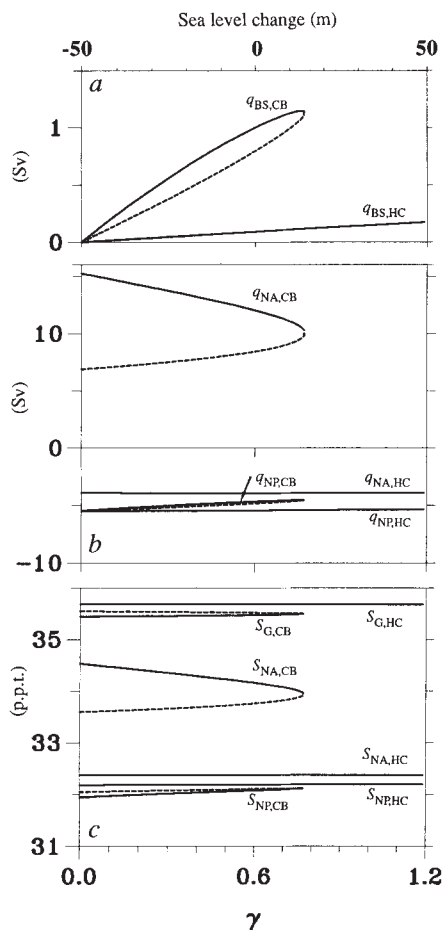


FIG. 2 Steady-state model solutions for the Bering Strait flow q_{BS} (a), the thermohaline flow in the North Pacific and North Atlantic oceans q_{NP} and q_{NA} (b), and the salinity in the North Pacific, North Atlantic and the rest of the global ocean S_{NP} , S_{NA} and S_G (c), as functions of γ , the Bering Strait flow parameter, and of sea level change relative to modern sea level. The forcing and choice of parameters are guided by our knowledge of the modern ocean-atmosphere system. Stable and unstable solutions are shown as solid and dashed lines respectively. HC and CB refer to the 'halocline catastrophe' and the 'conveyor belt' modes respectively. $V_{NP,NA} = 0.1V_m$, $V_G = 0.8V_m$, $S_m = 35$ p.p.t., $\alpha = 0.15 \times 10^{-3} \text{ } ^\circ\text{C}^{-1}$, $\beta = 0.8 \times 10^{-3} \text{ p.p.t.}^{-1}$, $v = 10$, $T_{NP,NA} = 5 \text{ } ^\circ\text{C}$, $T_G = 20 \text{ } ^\circ\text{C}$, $q_f = 1 \text{ Sv}$ and $\delta = 0.6$. The sea level change scale follows from $\gamma \propto H$, as discussed in the caption to Fig. 1, given a modern Bering Strait depth of 50 m and a modern flow through the strait of 1 Sv, a flow found in the model for $\gamma = 0.6$ and other parameters set as above. No attempt has been made to correct salinity values for changes in sea level as the dynamics of the model depend on salinity differences which remain essentially unchanged for small changes in S_m .

Canadian and Siberian rivers, may be sufficient to force a climate 'flipover'.

Recent ice core data¹ indicate that climate, as recorded in Greenland, was quite variable during the last interglacial (Eemian) period. The time structure of this variability—very rapid transitions embedded in timescales of hundreds to thousands of years—suggest on-off switching of the North Atlantic thermohaline circulation as a prime cause. In contrast, the present interglacial has enjoyed a quite stable climate. Global mean temperatures and mean sea level may have been $0\text{--}2 \text{ } ^\circ\text{C}$ and $0\text{--}7 \text{ m}$ higher, respectively, during the Eemian period^{10,24} than today. According to our model and discussion above, stronger flow through the Bering Strait (and a stronger hydrological cycle to be expected under such conditions) would increase the likelihood of a collapse of the 'conveyor belt' mode by making this circulation more sensitive to perturbations. A subsequent return to cooler conditions in the North Atlantic region would presumably lay the groundwork for the return of the 'conveyor belt' mode. Very recent comparisons of data from the GISP2 and GRIP ice cores show discrepancies during the Eemian period which may be due to ice flow, expected to be more prevalent at the GISP2 site^{25,26}. Although confirmation of climate instability during the Eemian will require more work, our Bering Strait mechanism remains a viable one for promoting rapid Northern Hemisphere cooling during warm, interglacial periods.

Significant climate variability on timescales of hundreds to thousands of years in the North Atlantic region is also indicated during glacial times with low sea level and the Bering Strait closed^{2,3,27}. Furthermore, a wealth of evidence from deep-sea sediment cores points toward generally weak flow of North Atlantic Deep Water during these times^{28,29}. We suggest that the meridional temperature-gradient effect discussed above lies at the root of this behaviour, as also suggested by coupled general circulation model results³⁰. Our model predicts, for the parameter range of Fig. 2 with the Bering Strait closed ($\gamma = 0$), that a reduction of the meridional temperature difference in the ocean by $1.1 \text{ } ^\circ\text{C}$, from our choice of $15\text{--}13.9 \text{ } ^\circ\text{C}$, would lead to the collapse of the 'conveyor belt'. Such a reduction is not at odds with reconstructions of climate during the last glacial maximum³¹.

We suggest that thermohaline 'flipovers' and associated climate fluctuations in the North Atlantic region are most likely to

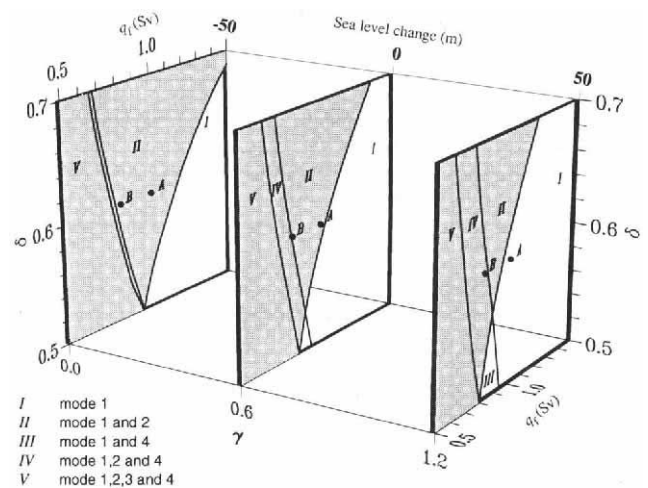


FIG. 3 Domains of stable, steady-state solutions of the model as functions of q_f , the fresh-water input, and δ , the fraction of q_f which enters the North Pacific, for three choices of γ , the Bering Strait flow parameter, and corresponding sea level change. All other constant and parameter choices are as for Fig. 2. Shaded areas mark the domains for which the 'conveyor belt' (mode 2) is a possible stable, steady-state solution. Mode numbers refer to the convention in the text. Point A corresponds to solutions presented in Fig. 2. Point B is discussed in the text.

occur when fresh-water forcing, temperature and/or sea level (Bering Strait 'openness') conditions are favourable for the collapse of the 'conveyor belt' ocean circulation mode. Such periods would be ice age terminations with strong melt water pulses, exceptionally warm interglacials with high sea level and a strong hydrological cycle, and glacial cold periods when meridional temperature forcing in the ocean is weak. Within this framework it is also possible to explain relative climate stability of intermediate situations like the present interglacial period and the early part of the last glaciation. □

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Maximum sea levels for the last glacial period from U-series ages of submerged speleothems

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PLEISTOCENE sea levels reflect changes in the surface distribution of land, sea and ice, which in turn significantly influence the Earth's climate¹. The most complete record of sea levels for the last glacial cycle has come from raised coral reef terraces of the Huon Peninsula, Papua New Guinea^{2–4}, but interpretation of this record is hampered by relatively large age uncertainties and the possibility of variation in the rate of tectonic uplift. Subaerial speleothems (stalagmites, stalactites and flowstones) from submerged caves in tectonically stable areas provide an alternative source of data^{5–8}, as their growth stops when rising sea levels flood the caves. Previous studies of speleothems have been limited by dating precision^{5,6} and sample availability^{5–7}. Here we present high-precision thermal-

ionization mass-spectrometric ²³⁰Th ages^{9–11} for a comprehensive suite of speleothem samples from the Bahamas. Our results provide a record of maximum elevation of sea levels for the period from 80 to 10 kyr before the present, for much of which sea levels are poorly known. We also find that regional palaeoclimatic change can be an important factor in the termination of speleothem growth.

Samples were obtained from underwater caves ('blue holes') on Grand Bahama and South Andros islands using experimental heliox mixed-gas rebreather units provided by Carmellan Research. This advanced diving technology, used for the first time in caves during this project, offered the potential for accessing depths up to 180 m below sea level (referred to as –180 m), well beyond the –60 m limit of air-diving. In practice, the underwater caves investigated could not be penetrated beyond boulder floors at –98 m. Speleothems were abundant to ~–45 m, but sparse beyond this depth. The deepest *in situ* sample collected was from –54.9 m, although *in situ* speleothems unsuitable for dating were observed at another site at –80 m. Sample elevations were determined using a digital depth gauge (± 0.1 m), corrected for water density, but not for semi-diurnal tides which have a maximum amplitude of 0.8 m. The area is tectonically stable, with long-term subsidence rates of the order of $2\text{--}3 \times 10^{-2}$ m kyr⁻¹ (refs 12, 13), so active speleothem growth, which is only possible in subaerial conditions, provides a precise constraint on maximum elevation of past sea levels.

Most samples were stalagmites with no significant post-depositional dissolution features and a single phase of growth, the initiation and cessation of which was initially dated by α -spectrometric ²³⁰Th methods. The large sample size required for this technique (50–100 g) resulted in time averaging, which was a particular problem in slow-growing samples. We therefore employed thermal-ionization mass spectrometry (TIMS) for precise determination of uranium and thorium isotope ratios in much smaller sub-samples, using a Finnigan-MAT 262-RPQ mass spectrometer, and analytical methods based on those described in refs 9–11. Precisions are similar to those previously reported^{7,10} (Fig. 1, Table 1), but sample sizes are an order of magnitude smaller. The majority of samples had very low levels of ²³²Th (²³⁰Th/²³²Th atomic ratios were $>5.4 \times 10^{-4}$, equivalent to an activity ratio of 100) and showed no evidence of diagenetic alteration. Duplicate and replicate analyses agreed within analytical precision, and consecutive stratigraphically related samples did not show age inversion, except in the case of GB-89-25-5C-CBT1, which contained some detrital material (²³⁰Th/²³²Th atomic ratio, 1.8×10^{-4}). We therefore believe that our age estimates are reliable.

In Fig. 2a, periods of continuous speleothem growth at a given depth are shown by continuous bars between basal (growth initiation) and top (cessation) age estimates. These define a constrained age/elevation sea-level curve whose structure is different to that from the raised coral-reef record in that it provides information on the maximum sea level for particular time ranges, rather than the estimated elevation and age of specific high stand events. Figure 2 shows the same general pattern of progressively lower sea levels towards the glacial maximum, followed by very rapid recovery on deglaciation well known from the $\delta^{18}\text{O}$ (refs 14, 15) and coral reef records^{2,4,14,16–19}. However, the depth dependence of the post-glacial cessation age apparent in Fig. 2 is not supported by statistical analysis if points with strong least-squares leverage are excluded (specifically that at 11.7 kyr, which is a significant outlier from the population of cessation ages). Nor can it be adequately demonstrated given the relatively high uncertainty of the α -spectrometric ages ($\sim \pm 2$ kyr), and limited depth coverage of mass spectrometric analyses (Table 1) available. More significantly, speleothem growth had stopped in samples well before rising sea levels^{18,19} could have inundated even the deepest samples. This suggests some control on speleothem growth other than sea level, a possibility not considered in previous studies^{5–7}.

TABLE 1 U and Th concentrations, isotopic ratios and U-series ages of Bahamian speleothems

Sample*	Sub-sample*	Elevation† (m)	$^{238}\text{U}^\ddagger$ (ng g ⁻¹)	$^{232}\text{Th}^\S$ (ng g ⁻¹)	$\delta^{234}\text{U}(\text{O})\parallel$ (‰)	$\delta^{234}\text{U}(\text{T})\parallel$ (‰)	$^{230}\text{Th}/^{238}\text{U}_{\text{act}}\P$	Age¶ (kyr)
AN-87-23-2	ABT1[1]	-53.6	324.9 ± 0.5	0.43 ± 0.01	-8.8 ± 1.7	-9.3 ± 2.5	0.1631 ± 0.0017	19.6 ± 0.2
	ABT1[2]		324.4 ± 0.6	0.43 ± 0.02	-11.0 ± 2.6	-11.7 ± 2.8	0.1608 ± 0.0028	19.3 ± 0.4
	ABT1[3]		325.6 ± 1.1	0.43 ± 0.01	-4.3 ± 3.7	-4.6 ± 3.9	0.1602 ± 0.0012	19.1 ± 0.2
	ABT1[4]		325.8 ± 0.8	0.43 ± 0.01	-6.4 ± 3.1	-6.7 ± 3.3	0.1605 ± 0.0012	19.2 ± 0.2
	ABT2[1]		343.7 ± 0.9	0.13 ± 0.03	-7.5 ± 2.4	-7.9 ± 2.5	0.1607 ± 0.0055	19.2 ± 0.7
	ABT3[1]		321.9 ± 0.7	0.12 ± 0.03	-4.1 ± 3.0	-2.7 ± 3.2	0.1609 ± 0.0046	19.2 ± 0.6
	ABB1[2]		478.0 ± 5.0	0.17 ± 0.01	3.9 ± 3.9	3.9 ± 4.1	0.1634 ± 0.0027	19.3 ± 0.4
AN-87-23-3	AAT1[1]	-50.5	282.9 ± 0.7	1.59 ± 0.01	40.6 ± 3.6	42.8 ± 3.8	0.1627 ± 0.0012	18.5 ± 0.2
	AAB1[1]		412.9 ± 0.7	0.14 ± 0.07	39.4 ± 2.1	41.9 ± 2.2	0.1886 ± 0.0013	21.8 ± 0.2
AN-87-23-6	AAT1	-49.1	535.2 ± 3.1	13.40 ± 0.08	24.4 ± 13.5	37.2 ± 20.6	0.7679 ± 0.0078	149.2 ± 5.2
	AAT2		480.0 ± 4.2	5.99 ± 0.01	31.7 ± 2.1	50.3 ± 3.4	0.8048 ± 0.0086	162.4 ± 4.1
AN-87-26-5	ABT1[1]	-46.2	279.9 ± 1.5	0.91 ± 0.11	56.5 ± 5.3	59.2 ± 5.5	0.1516 ± 0.0017	16.8 ± 0.2
	AAB1		338.3 ± 0.5	0.20 ± 0.01	52.1 ± 2.4	51.9 ± 2.5	0.1659 ± 0.0023	18.7 ± 0.3
AN-87-27-2	AAB1	-36.5	527.8 ± 7.0	0.34 ± 0.01	36.0 ± 10.4	40.2 ± 11.6	0.3148 ± 0.0046	39.3 ± 0.9
AN-87-28-1	AAT1[1]	-35.7	387.5 ± 0.2	2.21 ± 0.08	42.4 ± 1.8	47.4 ± 2.0	0.3211 ± 0.0024	39.9 ± 0.4
	AAT2[1]		444.1 ± 0.3	0.41 ± 0.09	41.3 ± 1.7	46.2 ± 1.9	0.3211 ± 0.0021	40.0 ± 0.3
	AAB1[1]		636.3 ± 0.3	0.59 ± 0.05	45.1 ± 1.2	50.6 ± 1.3	0.3263 ± 0.0015	40.6 ± 0.2
AN-87-31-1	AAB1	-25.6	732.4 ± 9.0	0.10 ± 0.02	35.8 ± 3.7	40.6 ± 4.3	0.3685 ± 0.0067	47.7 ± 1.1
GB-89-25-5A	CEB2	-18.1	232.6 ± 0.3	0.72 ± 0.02	52.5 ± 3.3	62.6 ± 4.0	0.4608 ± 0.0026	62.3 ± 0.6
	BEB1[2]		207.9 ± 0.1	1.76 ± 0.01	57.7 ± 2.7	72.0 ± 3.4	0.5451 ± 0.0026	78.1 ± 0.6
	CEB1[1]		207.3 ± 0.2	1.56 ± 0.02	54.6 ± 1.7	67.6 ± 2.5	0.5351 ± 0.0032	76.8 ± 0.5
	CEB1[2]		207.1 ± 0.1	1.56 ± 0.02	58.2 ± 1.3	71.3 ± 1.6	0.5329 ± 0.0031	76.0 ± 0.5
	BDT1		265.2 ± 0.2	0.30 ± 0.01	47.6 ± 3.0	72.7 ± 4.7	0.7886 ± 0.0028	149.2 ± 1.7
	DDT1[1]		279.1 ± 0.2	0.15 ± 0.01	44.5 ± 2.1	68.4 ± 3.3	0.7917 ± 0.0032	151.6 ± 1.5
	DDT1[2]		278.9 ± 0.1	n.d.	45.4 ± 2.0	n.d.	n.d.	n.d.
	DDT2		275.8 ± 0.2	0.08 ± 0.02	48.1 ± 2.7	73.2 ± 4.1	0.7864 ± 0.0029	148.2 ± 1.5
GB-89-25-5B	BGT1	-18.1	268.3 ± 0.5	1.90 ± 0.01	44.8 ± 3.1	69.9 ± 4.9	0.8044 ± 0.0038	156.8 ± 2.1
GB-89-25-5C	CBT1	-18.1	111.4 ± 0.9	2.99 ± 0.02	29.4 ± 8.1	32.8 ± 9.0	0.3013 ± 0.0050	37.6 ± 0.8
	CBT4[2]		142.6 ± 0.4	0.14 ± 0.02	49.3 ± 6.7	52.7 ± 7.2	0.2070 ± 0.0044	23.9 ± 0.9
	BBB1[A]		112.5 ± 0.2	0.19 ± 0.01	57.0 ± 7.6	67.1 ± 4.3	0.4352 ± 0.0058	57.4 ± 0.7
	BBB1[B]		154.2 ± 0.3	0.18 ± 0.02	54.5 ± 3.7	64.3 ± 4.3	0.4405 ± 0.0037	58.5 ± 0.7
	CAT1[2]		194.8 ± 3.0	0.22 ± 0.01	49.2 ± 7.9	59.0 ± 9.5	0.4669 ± 0.0082	63.7 ± 1.7
	BAB1		286.8 ± 0.6	2.58 ± 0.07	60.0 ± 5.3	75.1 ± 6.7	0.5522 ± 0.0082	79.4 ± 1.8
GB-89-25-6	AAT1	-13.8	184.2 ± 0.1	1.22 ± 0.04	45.7 ± 1.6	47.8 ± 1.6	0.1378 ± 0.0009	15.4 ± 0.1

* Speleothem samples were collected from submerged caves on the islands of Grand Bahama (GB) and South Andros (AN). [1]–[4] denotes analyses on sub-sample aliquots, those denoted [A], [B] are analyses of different fragments of the same sub-sample wafer.

† Elevations are in m (±0.1) below present sea level (no correction made for semi-diurnal tides and Pleistocene subsidence rates).

‡ ^{238}U concentration is calculated using $^{238}\text{U}/^{235}\text{U} = 137.88$.

§ ^{232}Th concentration is corrected for an analytical blank of $5 \pm 2.5 \text{ pg } ^{232}\text{Th}$.

|| $\delta^{234}\text{U} = \{[(^{234}\text{U}/^{238}\text{U})/(^{234}\text{U}/^{238}\text{U})_{\text{eq}}] - 1\}$, where $(^{234}\text{U}/^{238}\text{U})_{\text{eq}}$ is the atomic ratio at secular equilibrium and is equal to 5.472×10^{-5} . The $\delta^{234}\text{U}$ value thus obtained is then multiplied by 10^3 to yield units of ‰. $\delta^{234}\text{U}(\text{O})$ is the measured value. $\delta^{234}\text{U}(\text{T})$ is the initial value and is equal to $[\delta^{234}\text{U}(\text{O})]/[e^{(\lambda_{234}T)}]$, where λ_{234} is a decay constant (see below) and T is the age in years.

¶ Ages are calculated using $[^{230}\text{Th}/^{238}\text{U}]_{\text{act}} - 1 = e^{-\lambda_{230}T} + (\delta^{234}\text{U}(\text{O})/1,000)(\lambda_{230}/\lambda_{234} - \lambda_{234})(1 - e^{-(\lambda_{230} - \lambda_{234})T})$, where T is the age in years and $[^{230}\text{Th}/^{238}\text{U}]_{\text{act}}$ is the $^{230}\text{Th}/^{238}\text{U}$ activity ratio. Decay constants; $\lambda_{238} = 1.551 \times 10^{-10} \text{ yr}^{-1}$, $\lambda_{234} = 2.835 \times 10^{-6} \text{ yr}^{-1}$, $\lambda_{230} = 9.195 \times 10^{-6} \text{ yr}^{-1}$ (original refs in ref. 12). Errors are $2\sigma_{\text{mean}}$ or $2\sigma_{\text{counting statistics}}$, whichever is the greater.

Speleothem growth in subaerial caves may be stopped by reduction in the magnitude and duration of groundwater recharge (controlled by the effective precipitation and hydrological routing in the unsaturated zone overlying the cave), or lowering of the calcium content of drip waters, which is primarily dependent on the generation of carbonic acid by biological processes in the overlying soils. Many bedrock surfaces in the northern Bahamas preserve soil remnants (palaeosols)²⁰; however, there is no evidence that past soil coverage was more extensive than today²¹. Present-day soil CO_2 concentrations are sufficient for speleothem growth²², but observations in modern Bahamian caves show an almost complete absence of growth. This is due to the lack of active drip water, which may be explained by either the low present-day rate of recharge (precipitation is $\sim 1,300 \text{ mm yr}^{-1}$, approximately equal to potential evapotranspiration, although effective evaporation is 500 mm less), or more probably to the limited depth of the unsaturated zone overlying the caves (typically $< 5 \text{ m}$) which limits storage of storm rainfall and hence

continuous supply of water for cave drips. But the latter cannot explain the post-glacial cessation of speleothem growth, which occurred without change in the depth of the unsaturated zone overlying the sampled speleothems. We therefore suggest that the cessation results from palaeoclimatic change, from wetter conditions (during stages 3 and 2) to drier conditions which limited recharge.

Results from general circulation modelling (GCM) studies^{23, 25} for the last glacial maximum show that the North American jet stream bifurcated around the Laurentide ice sheet, the southern branch being strengthened and displaced further to the south. An associated steepening of the climatic gradient between the warm ocean and advected cold air from the ice sheet, caused a major increase in frontal rainfall along the eastern seaboard of North America and the Bahamas study area. This has been demonstrated by 18 kyr before present model simulations^{23, 25}, where estimates of annual mean net precipitation minus evaporation were up to $\sim 4 \text{ mm d}^{-1}$ greater than

present-day values. An abrupt change in regional climate occurred with the onset of deglaciation. Melt water from the Laurentide ice sheet discharged into the Gulf of Mexico and caused a reduction in regional sea surface temperatures (SST) and a high surface pressure anomaly, significantly suppressing precipitation. General circulation modelling incorporating a reduction of SST of 6 °C in the Gulf of Mexico and northern Caribbean at the time of maximum glacial meltwater input²⁶ has demonstrated negative anomalies of precipitation of up to 3 mm d⁻¹ relative to present-day values. Sensitivity analyses indicate that similar changes could result from much smaller meltwater-driven temperature changes²⁷. We believe that the cessation of speleothem growth before flooding by rising sea levels is related to these regional palaeoclimatic changes, which are also reflected in a

palynological record from Lake Tulane, Florida²⁸. Of the speleothems known to be growing after 20 kyr, 11 useful estimates of the age of cessation of growth were obtained by extrapolation. The mean age cessation is 17.1 ± 1.0 kyr ($2\sigma_{\text{mean}}$ excluding one outlier, 11.7 ± 1.2 kyr at -11 m) and provides an important chronological control for the regional drying associated with the onset of deglaciation. It is in good agreement with estimates for the start of post-glacial sea-level rise and lags a minimum of Northern Hemisphere insolation by ~5–6 kyr (ref. 29).

Preliminary data from three samples that record growth during isotope stage 6 (none of our samples were actively growing in stage 5) indicate that growth stopped between $149 \pm 5.2/-4.8$ kyr (latest growth for GB-89-25-5A&B and AN-87-23-6, Table 1) and 142 ± 3.2 kyr ($2\sigma_{\text{mean}}$ for equivalent sub-samples

FIG. 1 Plot of $\delta^{234}\text{U}(0)$ against $[^{230}\text{Th}/^{238}\text{U}]_{\text{act}}$ for Bahamas speleothem mass-spectrometric analyses (see Table 1 for definitions of these quantities). Contours of equal age are shown as straight lines with increasing slope. Also shown are contours of $\delta^{234}\text{U}(T)$. On a typical sub-sample of 150 kyr age which contains 50 ng of ^{238}U (1.3×10^{14} ^{238}U atoms, 7×10^9 ^{234}U atoms and 1.7×10^9 ^{230}Th atoms), routine precision (ellipses) is better than $\pm 0.5\%$ (2σ) for both $^{230}\text{Th}/^{238}\text{U}$ and $^{234}\text{U}/^{238}\text{U}$. Here we demonstrate the use of such analyses for determining the precise timing of events, shaded portions of the graph representing periods for which no growth has been found in this study.

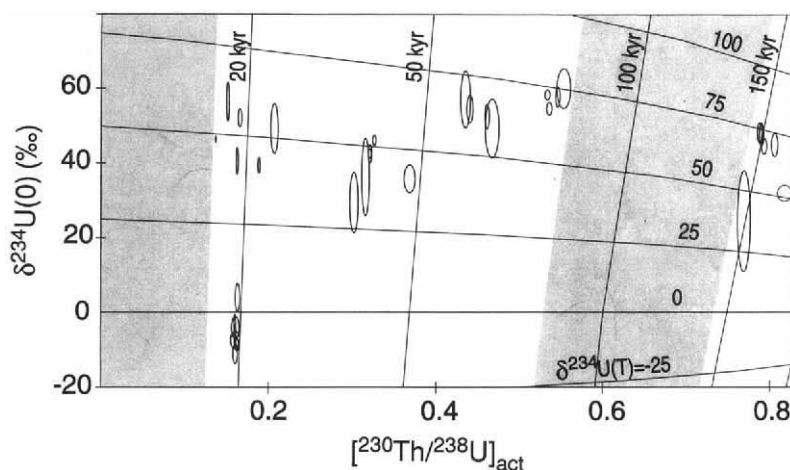
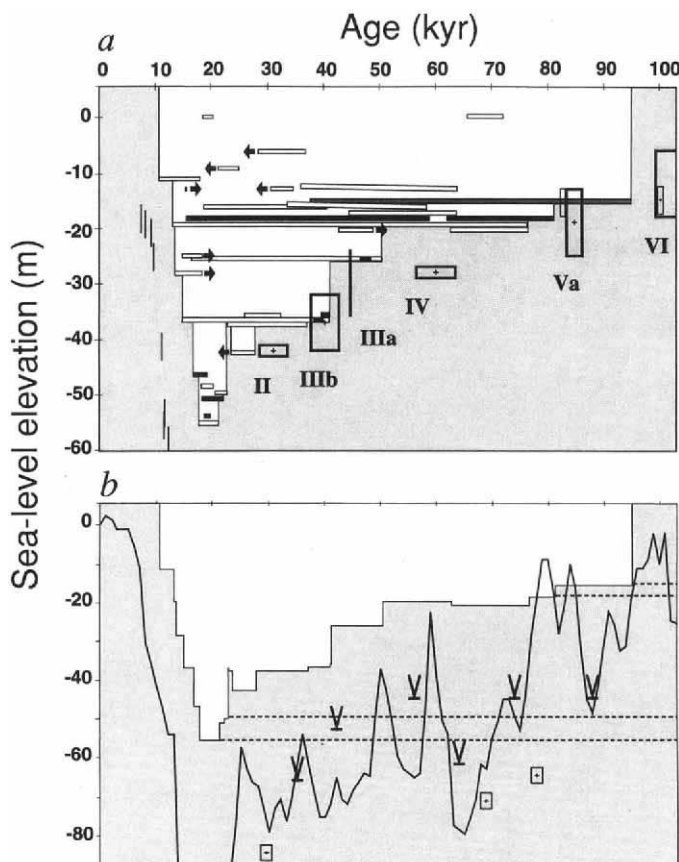


FIG. 2 Constrained age/elevation sea-level curve based on speleothems from the Bahamas. (Sea-level elevation defined as distance below present sea-level.) a, Horizontal bars represent single ages (arrows associated with bars indicate whether top or basal age only), or periods of continuous growth extrapolated from basal and top ages. Both α -spectrometric (this study, white bars) and mass-spectrometric analyses (this study, black bars, and ref. 7 grey bar) are shown. These bars constrain (± 0.1 m) the upper limit of sea-level elevations to within the shaded portion of the figure (subsidence rates of $2-3 \times 10^{-2}$ m kyr⁻¹ (refs 12, 13) are not taken into account). For comparison, estimates of age and elevation for high sea-stands, based on ^{230}Th ages of coral reef terraces are illustrated: Huon Peninsula II–VI (ref. 4) (α -spectrometric, 1σ , heavy boxes: age for IIIa reef crest is unknown and is based on the radiometric age of transgression phase, thus no age uncertainty) and Barbados^{33,38} (mass-spectrometric, 2σ , light boxes). Errors are those quoted in original references. We also show the post-glacial sea-level curve¹⁸ to highlight the cessation of speleothem growth before inundation by rising sea levels. b, Comparison with inferred record of ice volume from $\delta^{18}\text{O}$ of benthic foraminifera from the Norwegian Sea¹⁵ (—) and low sea-stands based on deltaic gravels from the Huon Peninsula¹⁴ (V) and dated corals below subaerial surface exposures, Barbados³⁸ (light boxes). Periods of time represented by hiatuses in growth for the stalactite sequence AN-87-23-6 and AN-87-23-2, and flowstone sequences GB-89-25-5A/B/C and DWBAH⁷ are also shown (---).



of DWBAH from ref. 7, although the surface of this sample is reported to have been truncated). By analogy with our results for the last glacial, we suggest that this early termination of speleothem growth may also be caused by increased regional aridity, synchronous with the onset of the penultimate deglaciation. If these inferences are correct, the onset of deglaciation occurs at a time of minimum insolation²⁹, as also suggested from the record of $\delta^{18}\text{O}$ in the Devil's Hole vein calcite³⁰. There is, however, considerable debate on the interpretation of the vein calcite record³¹, and further work is needed to confirm both the timing of the cessation of Bahamas speleothem growth and its dependence on regional palaeoclimate.

Speleothem growth is recorded at ~ -18 m for almost the whole glacial period. Although the flowstone sequence GB-89-25-5A/B/C from -18.1 m (Table 1, Fig. 1 and Fig. 2a) has a non-erosional hiatus between 63.7 ± 1.7 and 58.5 ± 0.7 kyr, other samples grew continuously throughout this period. We cannot, therefore, support the suggestions for high sea-stand events near present sea level³² between 93 and 15 kyr.

Figure 2 shows good correspondence between the speleothem and the Barbados sea-level record for the isotope stage 5a high-stand (Worthing Terrace), TIMS ^{230}Th age of 83.3 ± 0.3 kyr³³. Our sample from -18.1 m (GB-89-25-5A/B/C) started growth at 79.7 ± 1.8 kyr (based on extrapolation of the growth rate), whereas sample DWBAH⁷ from -15 m grew continuously from $92.6 \pm 2.6/-2.3$ kyr. The sea-level high is thus constrained to between -15 and -18 m, in remarkably good agreement with the elevation estimates derived from Barbados, -13 to -18 m (ref. 33), the estimate of 19 ± 5 m (1σ) from the Huon Peninsula⁴ (box in Fig. 1a), and that from Haiti of -13 ± 2 m (ref. 34). The general agreement observed between the four areas, all of which are minimally affected by differential isostatic effects due to ice loading³⁵, suggests that geoidal eustatic effects are probably less important than has been previously suggested³⁶. The age of 79.7 ± 1.8 kyr for initiation of growth after a hiatus in GB-89-25-5A/B/C is also important because it precisely constrains the termination of stage 5a, confirming the timing of this event (79.3 ± 3.6 kyr) derived from the orbitally tuned $\delta^{18}\text{O}$ record of Martinson *et al.*³⁷.

Comparative information for high sea-stands during stages 3 and 4 is only available from the Huon Peninsula⁴ (Fig. 2a), where rapid uplift has exposed a more complete sequence than on Barbados. Our constrained sea-level curve confirms the reliability of these high sea-stand elevations, suggesting that they are not affected by systematic or random variations in uplift rates. There is, however, a marked disparity between the Huon record (including low sea-stand events from deltaic gravel terraces^{3,14}) and the normalized $\delta^{18}\text{O}$ record from deep-sea cores¹⁵ for this period (Fig. 2b). In this respect, our speleothem data is somewhat disappointing. Two deep samples, AN-87-23-2 (-53.6 m) and AN-87-23-6 (-49.1 m) (Table 1, Fig. 1), show evidence of multiple growth phases, and one would expect speleothem growth to be represented for the periods of low sea level preceding Huon terrace II and IV high sea-stands (Fig. 2b). But despite extensive growth at shallower depths, none is recorded in either these or single phase samples from similar depths, during isotope stages 4 and 3. We do not know whether this is because of sample bias, a lag in commencement of growth or some other factor. Thus, while it is clear that precisely dated subaerial speleothem samples from submerged caves have the potential to define a 'forbidden zone' into which sea level cannot have risen, future studies must address the role of both palaeoclimate and other (unknown) factors which may also control the initiation and cessation of speleothem growth. □

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Oceanic mafic granulite xenoliths from the Kerguelen archipelago

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OCEANIC plateaus are large accumulations of volcanic and plutonic rocks on the ocean floor, whose detailed nature and origin are only poorly understood. Their immense volumes, frequent occurrence since the Cretaceous period¹ and conspicuous presence in all major ocean basins point to their significance in understanding the Earth's thermal and chemical evolution. We report here the discovery of mafic granulite xenoliths from Kerguelen island, at the northern end of the Kerguelen plateau—one of the two largest oceanic plateaus. Granulites are normally found only in continental settings, where the crust is thick enough to experience the high temperatures and pressures required for granulite-facies minerals

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to form. We speculate that the Kerguelen xenoliths represent basaltic magmas underplated during the evolution of the plateau, and more generally that such mafic granulites are an important constituent of the lower parts of oceanic plateaus, which are inaccessible by drilling.

The Kerguelen plateau, in the southern Indian Ocean, extends more than 2,000 km from 46° to 64° S, with a maximum width of 700 km (Fig. 1), and is the second largest oceanic plateau ($2.5 \times 10^7 \text{ km}^3$), after the Ontong Java plateau ($>5.0 \times 10^7 \text{ km}^3$ (ref. 2)). Its development is believed to have begun with the break-up of Gondwanaland 100–120 Myr ago^{3,7} and its evolution has been studied based on information derived from age dating, petrological and geochemical characteristics of volcanic rocks from the Bunbury basalts in Australia, Rajmahal Traps in India, Ninetyeast ridge, Broken ridge, Naturaliste plateau, as well as the Kerguelen islands, as these volcanoes are considered to be surface manifestations of the proposed Kerguelen plume at various stages^{4,8,10}. The structure and constitution of the plateau appear to be significantly different between the northern (46°–54° S) and southern (54°–64° S) parts. Based on information gained from seismic stratigraphy, Seasat altimetry and other geophysical observations, both parts are believed to have the characteristics of an oceanic crust; however the southern part is considered possibly to contain rifted, thinned continental crust, and to have experienced a complex history of subsidence, sedimentation and block faulting^{11,12}. Indeed, free-fall grab sampling in the Raggatt basin (56–60° S) recovered granitic and metasedimentary rocks of continental affinity¹² (although these could have been transported by icebergs), and the isotope systematics of rocks from Leg 119 Site 738 (in the extreme south of the plateau) suggest continental lithospheric contamination¹³. Drilling and dredging in the central part of the plateau (DSDP Leg 120, Sites 747, 749 and 750, 54°–59° S) produced transitional mid-ocean-ridge basalts (T-MORB) beneath Cretaceous sediments¹⁴.

The northern part of the plateau, which has Kerguelen island at its northern end, is believed to have formed while contiguous or near to Broken ridge 45 Myr ago, when the Kerguelen plume coincided with Southeast Indian ridge¹⁵ (a geodynamic setting similar to Iceland today¹⁶) and evolved more recently to an intraplate setting akin to that of Hawaii^{17–19}. The geochemical characteristics of rocks exposed on Kerguelen reflect this geodynamic evolution, as older (~40 Myr) tholeiitic-transitional basalts display $\varepsilon_{\text{Nd}} = +3$ and $^{87}\text{Sr}/^{86}\text{Sr} = 0.7048$, whereas younger (~26 Myr to present) alkaline basalts show $\varepsilon_{\text{Nd}} = -6$ and $^{87}\text{Sr}/^{86}\text{Sr} > 0.706$ (refs 9, 20).

Xenoliths in volcanic rocks provide unique information about the chemistry and mineralogy of basement rocks beneath volcanoes that cannot be sampled easily, and those from Kerguelen can be used to infer the basement structure and architecture of at least the northern part of the plateau. Numerous basic and ultrabasic xenoliths are found in pipes and dikes of highly alkaline rocks cutting across older plateau lavas²¹. Such occurrences

TABLE 1 Compositions of sapphirine and host xenoliths

	Sapphirine			Whole rock	
	MG91116	MG9199A		MG91116	MG9199A
SiO ₂	13.36	14.19	SiO ₂	44.54	47.38
TiO ₂	0.11	0.11	TiO ₂	0.03	0.04
Al ₂ O ₃	64.08	61.85	Al ₂ O ₃	27.59	30.74
Cr ₂ O ₃	0.54	1.06	FeO(t)	2.49	1.21
FeO	2.6	5.34	MgO	10.24	3.41
MgO	18.95	18.55	MnO	0.05	0.03
NiO	0.29	0.09	CaO	13.91	17.12
MnO		0.02	Ma ₂ O	1.08	1.19
CaO	0.05	0.04	K ₂ O		0.04
Na ₂ O	0.04		P ₂ O ₅	0.02	0.01
K ₂ O	0.04				
Cation content*			CIPW norms		
Si	0.779	0.828	C	0.57	
Ti	0.005	0.005	Or		0.17
Al	4.401	4.252	Ab	9.15	10.00
Cr	0.025	0.049	An	68.88	77.90
Fe ²⁺	0.127	0.261	DiFe		0.70
Fe ³⁺			DiMg	4.30	
Mg	1.646	1.613	HyFe	0.01	0.80
Ni	0.013	0.004	HyMg	0.08	4.00
Mn		0.001	Fa	2.9	0.40
Ca	0.003	0.003	Fo	17.83	1.50
Na	0.004		Mt	0.47	0.20
K	0.003		Ilm	0.06	0.03
			Ap	0.05	

Sapphirine analysed by electron microprobe, whole rock analysed by X-ray fluorescence, results in wt%. CIPW norms represent an idealized mineralogical composition (wt%) calculated from chemical analysis of the rock. C, corundum; Or, orthoclase; Ab, albite; An, anorthite; DiFe, Fe-diopside; DiMg, Mg-diopside; HyFe, Fe-hypersthene; HyMg, Mg-hypersthene; Fa, fayalite; Fo, forsterite; Mt, magnetite; Ilm, ilmenite; Ap, apatite. Other significant minerals for P–T estimates are the clinopyroxene 2 with Mg# (see text) from 87 to 92, orthopyroxene with Mg# from 84 to 92, spinel with Cr# = $\text{Cr}/(\text{Cr} + \text{Al})$ from 0.02 to 0.03, garnet with Mg# = 83 and the plagioclase from An₈₃ to An₈₇. MG 91-116 is a garnet + sapphirine-bearing granulite, MG 91-99A is a sapphirine-bearing garnet-free granulite.

* Per formula unit (10 oxygens).

in the Jeanne d'Arc peninsula (Fig. 1) contain basic granulites with granoblastic textures and mineral assemblages typical of the granulite facies: clinopyroxene + orthopyroxene + plagioclase + spinel ± garnet. Some garnet-free samples were described as spinel gabbros²². Granulite xenoliths have never before been reported from oceanic islands. Garnet-bearing mafic xenoliths do occur (for example, Salt Lake Crater, Hawaii²³), but are considered to be high-pressure cumulates from alkaline magmas²⁴. Plagioclase-bearing xenoliths are typically igneous-textured gabbroic textures without orthopyroxene²⁵.

In some of the granulite xenoliths with the Mg# (= $100 \text{ Mg}/(\text{Mg} + \text{Fe})$) > 73 and with high Al₂O₃ and CaO, sapphirine $[(\text{Mg}, \text{Fe})_{8-x}(\text{Al}, \text{Fe})_x]^{VI}[\text{Al}_x \text{Si}_{6-x}]^{IV}\text{O}_{20}$, where $4 \leq x \leq 5$ was

FIG. 1 The Kerguelen plateau (left) and the Kerguelen island (right). Symbols on the left-hand map have the following meanings; K, Kerguelen islands; H, Heard and MacDonald islands; NKP, northern Kerguelen plateau; SKP, southern Kerguelen plateau; solid black squares, seismic refraction profiles on the Kerguelen plateau, with the diagonals of the squares corresponding to the profiles⁴⁰; white star in solid black circle, dredged Cretaceous cherts⁴¹. Symbols on the right-hand map have the following meanings; bold lines, seismic refraction profiles³⁴; black stars, location of the xenoliths referred to in this paper.

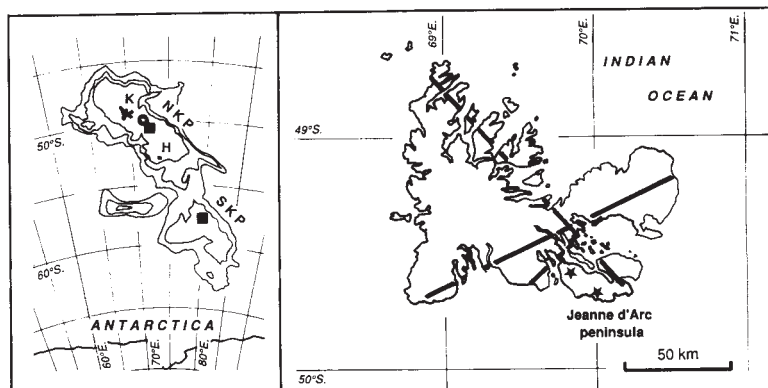


TABLE 2 Summary of the geothermometric data from field samples

Assemblage	Method	No. of samples	No. of mineral pairs	T(°C) average	T(°C) range
cpx-opx-pl-sp-gt	Wells, opx/cpx ²⁸	2	5	920	900–970
cpx-opx-pl-sp-gt	Ellis & Green, cpx/gt ²⁹	2	5	970	910–1000
cpx-opx-pl-sp-gt	Harley, opx/gt ³⁰	2	5	930	890–980
cpx-opx-pl-sp-gt-sa	Wells, opx/cpx ²⁸	2	7	985	950–1000
cpx-opx-pl-sp-gt-sa	Ellis & Green, cpx/gt ²⁹	2	6	970	950–990
cpx-opx-pl-sp-gt-sa	Harley, opx/gt ³⁰	2	5	965	920–1010
cpx-opx-pl-sp	Wells, opx/cpx ²⁸	6	12	920	880–970
cpx-opx-pl-sp-sa	Wells, opx/cpx ²⁸	3	7	925	860–960
				P(GPa) average	P(GPa) range
cpx-opx-pl-sp-gt	Harley, opx/gt ³¹	2	5	1.2	0.85–1.50
cpx-opx-pl-sp-gt	Harley & Green, opx/gt ³²	2	5	1.1	0.75–1.45
cpx-opx-pl-sp-gt-sa	Harley, opx/gt ³¹	2	6	1.3	1.00–1.60
cpx-opx-pl-sp-gt-sa	Harley & Green, opx/gt ³²	2	6	1.1	0.80–1.45

Abbreviations used: cpx, clinopyroxene; opx, orthopyroxene; pl, plagioclase; sp, spinel; gt, garnet; sa, sapphire.

found in symplectitic intergrowth with garnet, clinopyroxene and rarely plagioclase: it was also found in coronitic reaction zones with clinopyroxene, spinel, $\pm$ garnet. Sapphirine generally occurs in granulite-facies metamorphic rocks in continental crust, and this seems to be the first report of its occurrence in an oceanic environment. In a typical coronitic occurrence in sample MG91-116 (Table 1), for instance, sapphirine occurs surrounding spinel, and is in turn surrounded by garnet. This occurrence has a striking petrographic resemblance to a kimberlite-borne granulite xenolith from Stockdale, Kansas²⁶.

The major-element composition of the sapphirine-bearing granulite (Table 1) implies protoliths rich in calcic plagioclase and high-Mg minerals. MG91-116 is a normative troctolite with calcic plagioclase and high-Mg olivine, whereas MG91-99A can be compositionally termed an anorthositic gabbro. Rare earth element (REE) abundance patterns of clinopyroxene, garnet, sapphirine and plagioclase in MG91-116 (determined by an ion microprobe at Woods Hole Oceanographic Institution using techniques described previously²⁷) are characterized by uniformly low overall abundance levels (clinopyroxene has 0.7–1 times chondrite for La to Sm, 2 times chondrite for Eu, and lower concentrations for heavy REE with 0.2 times chondrite for Yb; garnet has 0.25–2 times chondrite for La to Sm, 5 times chondrite for Eu, and 0.6 times chondrite for Yb), and positive Eu anomalies in all mineral phases. All our data are consistent with a troctolitic protolith. Detailed accounts of mineral and whole-rock geochemistry will be published elsewhere.

Pressure-temperature conditions estimated using various thermobarometric methods^{28–32} range from 0.6 GPa, 900 °C to 1.6 GPa, 1,000 °C (Table 2), corresponding to depths of ~20–45 km (Fig. 2). The stability of sapphirine considered in the CaO–MgO–Al₂O₃–SiO₂ (CMAS) system involves a quartz-absent invariant assemblage of clinopyroxene + orthopyroxene + spinel + plagioclase + sapphirine + garnet around 1.0 GPa, 900 °C (ref. 33), which is in general agreement with the above estimated conditions. However, the textural relationships observed are not sufficient to precisely constrain P–T paths of the granulites. It may be suggested, nevertheless, that a significant increase in depth must have been experienced by MG91-116, if the protolith was indeed calcic plagioclase + forsteritic olivine as indicated by the bulk composition. On the other hand, there are numerous pyroxene granulites with well developed spinel-two-pyroxene symplectites, which could be produced simply by isobaric cooling of an olivine-plagioclase assemblage at relatively low pressures.

The existence of granulites beneath Kerguelen offers a new interpretation of the geophysical data. Seismic refraction studies of the northern part of the Kerguelen plateau³⁴ have established that a 8 to 9-km-thick layer with average compressional wave velocity (V_p) of 5.5 km s⁻¹ (identified as oceanic layer 2) is

underlain by oceanic seismic layer 3 with V_p = 6.6 km s⁻¹ extending to depths of 14–17 km. Beneath this apparently more typical, but thickened oceanic crust, a zone with V_p = 7.2–7.5 km s⁻¹ is recognizable and the crust–mantle boundary cannot be modelled as a single step. The 'low-velocity' region is interpreted as either serpentinized mantle peridotites or underplated magmas. Following the idea proposed for rifted continental margins^{35,36}, for continental flood basalts², and for the Kerguelen plateau, it was argued that the observed V_p = 7.2 km s⁻¹ could be consistent with underplating of large quantities of picritic melt with ~16 wt% MgO (ref. 34).

Based on the compressional wave velocities measured in minerals in the laboratory (see, for example, Table 2 of ref. 35), plagioclase-bearing mafic rocks such as granulites are consistent

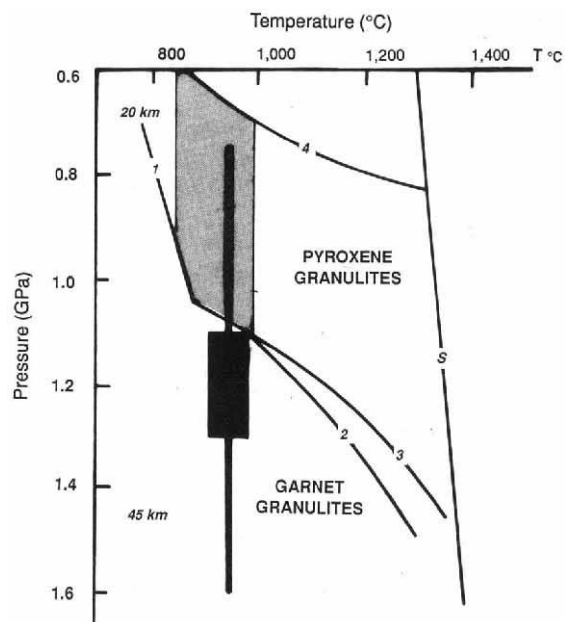


FIG. 2 P–T estimates for sapphirine-bearing granulites from Kerguelen. The dark-shaded area represents the average range of P–T conditions measured on garnet-bearing granulites with deviations given by the bold lines. The light-shaded area (in the pyroxene granulite field of the CMAS system³²) indicates the range of temperature calculated for Kerguelen garnet-free granulites. Univariant reaction curves as follows: 1, orthopyroxene + plagioclase + spinel → sapphirine + clinopyroxene; 2, orthopyroxene + spinel + plagioclase → sapphirine + garnet; 3, orthopyroxene + spinel + plagioclase → clinopyroxene + garnet; 4, olivine + plagioclase → orthopyroxene + clinopyroxene + spinel. S indicates basalt solidus.

with the observed range of V_P (7.2–7.5 km s⁻¹). For instance, V_P calculated for a garnet granulite consisting of plagioclase (40 wt%), clinopyroxene (30%), orthopyroxene (10%) and garnet (20%) is 7.38 km s⁻¹, and plagioclase (6.166 km s⁻¹) and garnet (8.921 km s⁻¹) have opposing effects on calculated V_P of rocks. Occurrences of mafic granulites as xenoliths are abundant in Kerguelen island. Indeed, in addition to the localities in the southeastern part of the islands, at least four localities were discovered and sampled in recent field seasons in the northeastern province, the Courbet peninsula. They indicate that a significant volume of granulite could exist beneath the islands and the northern Kerguelen plateau. The existence of a 'low-velocity mantle' zone beneath oceanic islands has been recognized beneath Marquesas³⁷, and the progressive changes in seismic velocities have also been proposed for the Madagascar ridge³⁸ and the Crozet plateau³⁹. Based on our finding, we speculate that mafic granulites can account for the observed seismic characteristics, and thus can be important constituents in those oceanic areas where large-scale magma production is predicted. If granulites represent underplated basaltic magmas and cumulates therefrom, which are otherwise not sampled by subaerial or submarine volcanics, their trace element and isotopic characteristics and geochronology are fundamentally important for understanding of the evolution of oceanic plateaus. □

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Biodiversity and stability in grasslands

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ONE of the ecological tenets justifying conservation of biodiversity is that diversity begets stability. Impacts of biodiversity on population dynamics and ecosystem functioning have long been debated^{1–7}, however, with many theoretical explorations^{2–6,8–11} but few field studies^{12–15}. Here we describe a long-term study of grasslands^{16,17} which shows that primary productivity in more diverse plant communities is more resistant to, and recovers more fully from, a major drought. The curvilinear relationship we observe suggests that each additional species lost from our grasslands had a progressively greater impact on drought resistance. Our results support the diversity–stability hypothesis^{5,6,18,19}, but not the alternative hypothesis that most species are functionally redundant^{19–21}. This study implies that the preservation of biodiversity is essential for the maintenance of stable productivity in ecosystems.

The resistance of an ecosystem to perturbation and the speed of recovery, which is called resilience, are two important components of ecosystem stability⁶. Interest in the effects of biodiversity on stability has been heightened by the rapidly accelerating rate of species extinctions^{18,22,23}. One view, the diversity–stability hypothesis, holds that species differ in their traits and that more

diverse ecosystems are more likely to contain some species that can thrive during a given environmental perturbation and thus compensate for competitors that are reduced by that disturbance^{5,6,7,12,18,19}. This view thus predicts that biodiversity should promote resistance to disturbance. In contrast, the species-redundancy hypothesis asserts that many species are so similar that ecosystem functioning is independent of diversity if major functional groups are present^{19–21}. An 11-year study of the factors controlling species composition, dynamics and diversity in successional and native grasslands in Minnesota^{16,17} provides a test of the effects of biodiversity on ecosystem response to and recovery from a major perturbation. The study period included the most severe drought of the past 50 years²⁴ (1987–88), which led to a >45% reduction in above-ground living plant mass and a >35% loss of plant species richness in control plots²⁴.

Nitrogen is the major nutrient limiting productivity in most terrestrial habitats²⁵, including these Minnesota grasslands¹⁶. The species composition, diversity and functioning of these and many other ecosystems depend on the rate of nitrogen supply^{17,25,26} and are thus being altered by increased atmospheric nitrogen deposition from agriculture and combustion of fossil fuels^{27–29}. In 1982 we established, in four grassland fields, a total of 207 control and experimental plots in which plant species richness was altered through seven different rates of nitrogen addition¹⁶.

We measured resistance to drought by calculating, for each plot, the relative rate of plant community biomass change ($\delta B/Bdt$, yr⁻¹; Fig. 1) from 1986, the year before the drought, to 1988, the peak of the drought. Values closer to zero imply greater drought resistance. For our 207 plots, drought resistance was a significantly ($P < 0.0001$) increasing, but saturating, function of pre-drought plant species richness (Fig. 1). The greatest dependence of drought resistance on species richness occurred in plots

with nine or fewer species. The most species-rich plots produced about half of their pre-drought biomass during the drought, whereas the most species-poor plots produced only about one-eighth (Fig. 1).

Other characteristics of plots, such as the rate of nitrogen addition, total above-ground plant biomass, the proportion of total plant biomass from species with the C4 photosynthetic pathway, and differences in these variables among fields, were also correlated with species richness. Species composition and abundances also varied with species richness in these plots^{16,17}. More than 90% of the plots that contained four or fewer plant species were dominated (>50% of plot biomass) by *Poa pratensis*, *Agropyron repens* or *Schizachyrium scoparium*. *Poa* and *Agropyron* are drought-sensitive C3 grasses, and *Schizachyrium* is a drought-resistant C4 grass. A partial correlation analysis that controlled for all of these potentially confounding variables (including the 1986 biomasses of these three species) showed a significant dependence of drought resistance on the natural logarithm of pre-drought species richness ($r_{\text{partial}} = 0.21$, $n = 207$, $P < 0.01$). Moreover, when all redundant, nonsignificant variables were removed using backwards elimination, species richness was retained, and its partial correlation with drought resistance was highly significant (Table 1).

The dynamics of individual species in our plots suggest that species richness led to greater drought resistance because species-

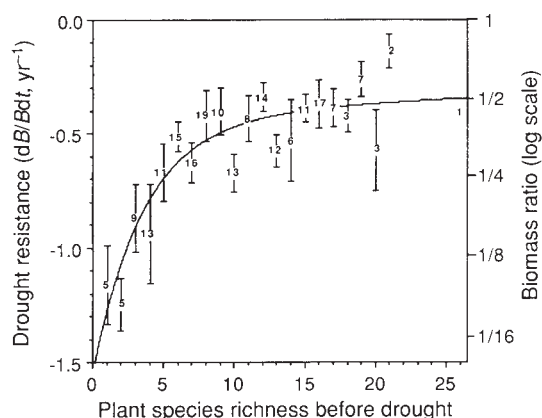


FIG. 1 Relationship between drought resistance of grassland plots and plant species richness (SR_{86}) preceding a severe drought. Mean, standard error and number of plots with a given species richness are shown. Drought resistance was measured as dB/Bdt (yr^{-1}), that is, as $0.5 \ln [biomass_{1988}/biomass_{1986}]$, where $biomass_{1988}$ is at the height of drought and $biomass_{1986}$ is for the year preceding drought. Biomass ratio ($biomass_{1988}/biomass_{1986}$; right-hand scale) shows the proportionate decrease in plant biomass associated with the dB/Bdt values. Because the correlation between species richness and drought resistance in our data was no longer significantly ($P \leq 0.05$) positive when all plots with ≤ 8 or ≤ 11 species were ignored, we cannot reject the hypothesis that the relationship may reach a plateau. The solid curve ($dB/Bdt = -1.13e^{-x/3.6} - 0.44e^{-x/110}$, where x is SR_{86} , $r^2 = 0.22$, $P < 0.0001$), which was fitted to all 207 data points, is simply one of many that gave a significantly better fit than a straight line. A simpler equation ($dB/Bdt = 0.304 \ln [SR_{86}] - 1.21$; $r^2 = 0.21$, $P < 0.0001$) provided an equally good fit.

METHODS. These are described in detail in ref. 16. The 207 plots were located in existing vegetation in four grassland fields in Cedar Creek Natural History Area, Minnesota. Field A had been abandoned for 20 yr, field B for 31 yr and field C for 54 yr in 1988. Each contained 54 plots, each 4×4 m. Field D, a prairie opening in native savannah, contained 45 plots, each 2×4 m. Nine treatments: no nutrient addition, addition of macro- and micro-nutrients other than nitrogen, and seven treatments that received these nutrients but with seven different rates of nitrogen addition. Field D had five replicates per treatment, the others had six. Vegetation in each plot was sampled by clipping a different 0.3-m^2 subsection each year, sorting to species, drying and weighing. Species richness is the number of vascular species in a 0.3-m^2 sample. Biomass is total above-ground living plant mass ($g\text{ m}^{-2}$).

TABLE 1 Factors influencing drought resistance

Variable	Partial correlation coefficient	P
Field A intercept	-0.56	<0.0001
Biomass of <i>P. pratensis</i> (1986)	-0.39	<0.0001
Biomass ₁₉₈₆	-0.36	<0.0001
$\ln (SR_{1986})$	0.29	<0.0001

Partial correlation of each listed variable with dB/Bdt , controlling for the other listed variables. Analyses used backwards elimination in multiple regression analyses to retain only variables that had significant ($P < 0.05$) partial correlations with dB/Bdt . The final multiple regression was highly significant ($F = 48.8$, $n = 207$, $R^2 = 0.48$, $P < 0.0001$). Extensive residual analyses³⁰ were performed at each step. Candidate variables included $\ln (SR_{1986})$, logarithm of experimental plus atmospheric nitrogen addition, $biomass_{1986}$, number of species of C3 and C4 plants in each plot, fraction of biomass comprised of C3 and C4 plants, dummy variables³⁰ for each field, and the biomass in 1986 of the three most common species in low species-richness (one to four species) plots (*A. repens*, *P. pratensis* and *S. scoparium*). The significant partial correlation for $\ln (SR_{1986})$ means that the correlation of species richness with drought resistance does not arise through biomass, species composition or field effects³¹. Other multiple regressions examined change in species richness and biomass preceding drought, detrended annual variation in species richness and biomass preceding drought, and their interactions with SR_{1982} and $biomass_{1982}$. No other multiple regression was significantly better than this one and all showed that partial correlations between species richness and drought resistance were statistically significant. Analyses using other measures of species diversity yielded similar results.

rich plots were more likely to contain some drought-resistant species. During this two-year drought, the increased growth of these drought-resistant species partially compensated for the decreased growth of other species.

A second component of stability is resilience, or the rate of return to pre-existing conditions after perturbation⁶. We calculated the deviation from pre-drought biomass as the natural logarithm of the ratio of plot biomass in 1989, 1990, 1991 and 1992 to average pre-drought biomass. For each of the four post-drought years, there were significantly negative intercepts and significantly positive slopes for regressions of these deviations on the

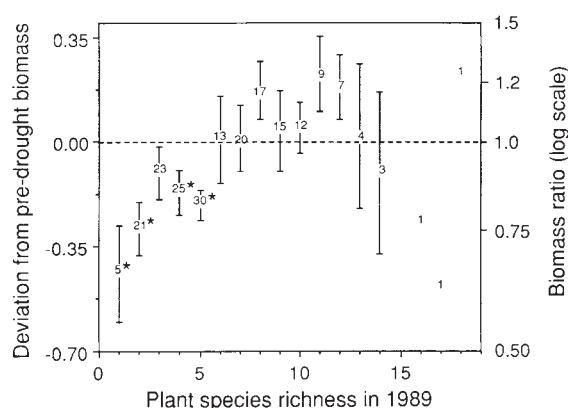


FIG. 2 Deviation of 1992 biomass from average (1982-1986) pre-drought biomass was measured as $\ln [(biomass_{1992})/(\text{average pre-drought biomass})]$. Mean, standard error and number of plots are indicated for each level of species richness. Negative values mean that 1992 biomass was lower than pre-drought average. Biomass ratio is ($biomass_{1992}/(\text{average pre-drought biomass})$). Student's *t*-tests showed that plots containing 1, 2, 4 or 5 species differed significantly ($* P < 0.05$) from their pre-drought average, but that plots with greater richness did not differ significantly from pre-drought averages.

TABLE 2 Factors influencing drought resistance

Variable	Partial correlation coefficient	P
Field A intercept	-0.39	<0.0001
Field B intercept	-0.30	<0.0001
Field C intercept	-0.20	0.003
ln(SR ₁₉₈₉)	0.18	0.009
SR _{C3}	-0.18	0.012
Biomass of <i>Schizachyrium</i> (1989)	-0.16	0.027
SR _{C4}	-0.14	0.042

Partial correlations of each listed variable with deviation from pre-drought biomass, holding other listed variables constant. These seven variables were retained in multiple regression analysis of 1992 deviation from pre-drought biomass against the same candidate variables used in Table 1, but using 1989 values. Backwards elimination, with residual analysis, was used to retain only significant ($P < 0.05$) variables. The overall regression had $F = 14.0$, $n = 206$, $R^2 = 0.33$, $P < 0.0001$. SR_{C3} is the number of C3 species and SR_{C4} is the number of C4 species in plots in 1989. The significantly positive slope for ln(SR₁₉₈₉) and the significantly negative intercepts for fields A, B and C indicate that species-poor plots in these fields have not yet attained pre-drought biomass, whereas more species-rich plots have. Field D, a native grassland, recovered most rapidly, followed by field C, then B, then A, in order of successional age.

natural logarithm of 1989 species richness. These indicate that species-poor plots were still further from their pre-drought biomass than were species-rich plots in each of the four post-drought years.

By 1992, species-rich plots had returned to pre-drought biomass, but the most depauperate plots still had significantly less biomass than their pre-drought average (Fig. 2). When potentially confounding variables were controlled for, there was a significant partial correlation between drought recovery and the natural logarithm of 1989 species richness ($r_{\text{partial}} = 0.184$, $P < 0.01$, $n = 207$). Moreover, when all redundant, nonsignificant variables were removed, species richness was retained, and its partial correlation was highly significant (Table 2). Thus, species-poor plots were both more greatly harmed by drought (Fig. 1 and Table 1) and took longer to return to pre-drought conditions (Fig. 2 and Table 2). The stand of native prairie was significantly more resilient than the three successional grasslands (Table 2).

Our results and earlier studies^{5,12,14,15} support the diversity-stability hypothesis⁵, and show that ecosystem functioning is sensitive to biodiversity. Our results do not support the species-redundancy hypothesis because we always found a significant effect of biodiversity on drought resistance and recovery even when we controlled statistically for the abundances of C3 (often drought sensitive) and C4 (often drought resistant) plant functional groups (Table 1).

Our results show that ecosystem resistance to drought is an increasing but nonlinear function of species richness. This is expected from the mechanism underlying the diversity-stability hypothesis. Functional diversity should be a saturating function of species richness because, in species-rich ecosystems, additional species are more likely to be similar to existing species²¹. Thus, the progressive loss of species should have progressively greater impacts on ecosystem stability.

In addition to drought, grassland ecosystems experience periodic invasions of insect or mammalian herbivores, unusually late or early frosts, unusually wet or cool years, hail, fire, and other perturbations. Because different species are likely to perform best for particular combinations of these disturbances, the long-term stability of primary production in these and other¹² grasslands should depend on their biodiversity. Although we do not know how the stability of other ecosystems depends on

biodiversity, these results lend further urgency to pleas for the conservation of biodiversity. □

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The role of partial occlusion in stereopsis

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MODELS of stereopsis typically assume that all the information about stereoscopic depth is contained in the disparity field, that is, the positional differences of image features that arise from surfaces visible to both eyes. But such models have difficulty in resolving image regions containing occlusions, because a portion of the occluded surface is visible to only one of the two eyes ('half-occlusions')¹. Here I present displays revealing an unexpected relationship between interocular differences in image position and occluding contours. The partial occlusion of contours can give rise to both horizontal and vertical image differences that are not disparities. The results show that the visual system interprets these image differences as signalling the presence of occluding contours. Even when a single line segment serves as a binocular target, subjective contours form that can appear both oriented and in depth. These local subjective contours have a strong tendency to interact cooperatively and form global contours not present in the monocular images. These and other findings^{2–4} show that stereoscopic processing actively decomposes vertical and horizontal image differences into disparities and half-occlusions. The two sources of information are complementary: while disparity provides relative depth information about surface features visible to both eyes, half-occlusions provide information to segment the visual world into coherent objects at object boundaries.

Previous theories of stereopsis have emphasized the relationship between interocular image displacements and binocular disparity. Indeed, the experience of stereoscopic depth was usually attributed to only the horizontal component of the disparity field^{5,6}. Mayhew and Longuet-Higgins⁷ challenged this view with a computational theory which demonstrated that the horizontal gradient of the vertical disparity field could be used to recover properties such as gaze angle and the absolute distance of surface features. Recent psychophysical experiments have revealed, however, that such gradients are either not used⁸ or contribute less information than is theoretically obtainable⁹.

One possible reason for the limited applicability of the Mayhew and Longuet-Higgins theory to human vision is that vertical image differences do not always represent vertical disparities. Indeed, occluding contours can generate both horizontal and vertical image displacements that are due to the presence of half-occlusions, not binocular disparities. This suggests a new way in which interocular differences in vertical position may influence stereoscopic depth, that is, by signalling the presence of an occluding contour. I present evidence here to support this idea. Figure 1a shows an example. When Fig. 1a is fused, observers report the appearance of an illusory window that is in front of and surrounds the line segments. This illusory window is also evident when the density of the lines is reduced (Fig. 1b, c). The illusions portrayed in Fig. 1 were created by lengthening the line segments in one of the two eyes. Hence, the subjective

contours (SCs) which formed are due solely to vertical image differences.

In theory, there are at least two ways that Fig. 1 could be perceived by the visual system. I will focus on one half of the stereopair in Fig. 1a to simplify the analysis of these patterns (Fig. 2a). One possibility is that the line terminators are matched, generating vertical disparities. Vertical disparities of this kind should cause the left and right halves of Fig. 1a to appear slanted in stereoscopic depth about a vertical axis of rotation, as has been shown previously for patterns such as random dot stereograms (known as the 'induced effect'; ref. 10; see

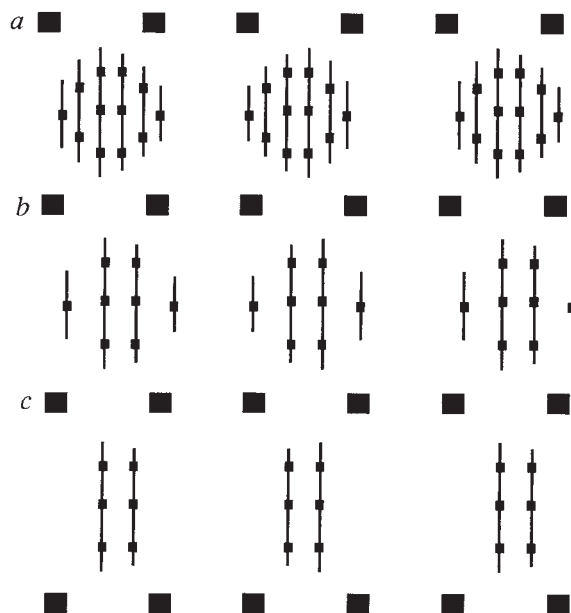


FIG. 1 Examples of the formation of subjective contours by interocular differences in vertical position. Cross-fusers should fuse the left two columns, divergers or viewing with a stereoscope should fuse the right two columns. In this and subsequent figures, the description of the SCs only holds for the pattern in which the line segments appear behind the small squares in the surround. In the central pattern of a, the rightmost three lines are slightly longer on both ends of the figure than the corresponding lines in the other eye's view. Similarly, the leftmost three lines of the central figure are smaller than the corresponding lines in the other eye's view. When fused, observers report the appearance of a compelling illusory 'window' that forms in front of the line segments as part of an apparent occluding surface. An occluding surface of this kind would produce the pattern of vertical image displacements depicted. The illusory window is apparent even when the density of the line segments is reduced to four (b) or two (c) line segments. The large squares in the surround and the small squares on the line segments in this and subsequent figures prevented eye and head movements from eliminating the vertical displacements of the line targets. They also served as reference disparities appropriate to the depth of the illusory window and line segments, respectively.

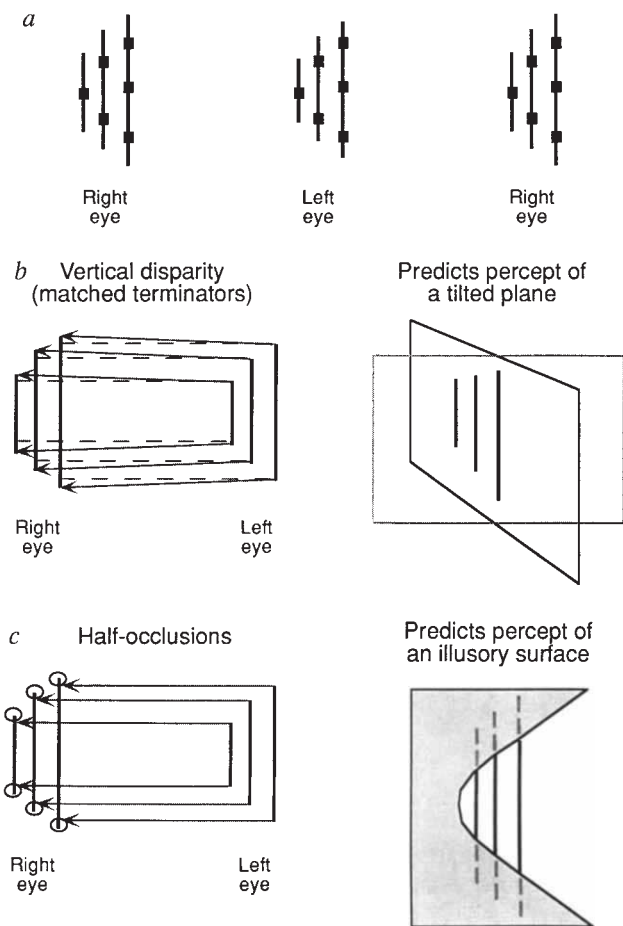


FIG. 2 Diagram of the possible interpretations of vertical image displacements for the left and right eye patterns depicted in a. In this stereopair, the line segments in the right eye's view are longer on the top and bottom than the left image. b, Diagram of the percept expected if the line terminators are matched, generating vertical disparities. In the left half of the figure, dashed lines served as references to depict the difference in height of the terminators in the two eyes; arrows indicate that the terminators are matched. The lines of the stereogram should appear to lie in a plane tilted in depth, as has been shown previously for patterns such as random-dot stereograms (known as the 'induced effect')⁷. This possible percept was shown to 33 observers, but none reported this experience. c, Diagram of the percept expected if the 'extra' line lengths in the right eye's view (terminators enclosed by circles) are treated as half-occlusions, that is, monocular features that arise from binocularly viewing scenes containing occluding surfaces. The grey region depicts the percept of an occluding surface that is in front of the line segments. No difference in the apparent luminance of these regions was reported and is presented only for schematic purposes. Note that this pattern of monocular features can only arise in natural viewing conditions if occluding surfaces are present. In these stereopairs, however, the occluding contours are not visible monocularly, but are created by interpreting the line terminators of the right eye as half-occlusions. This percept was observed by all subjects tested ($n = 33$; $P < 0.001$).

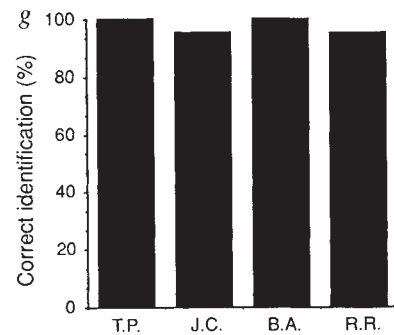
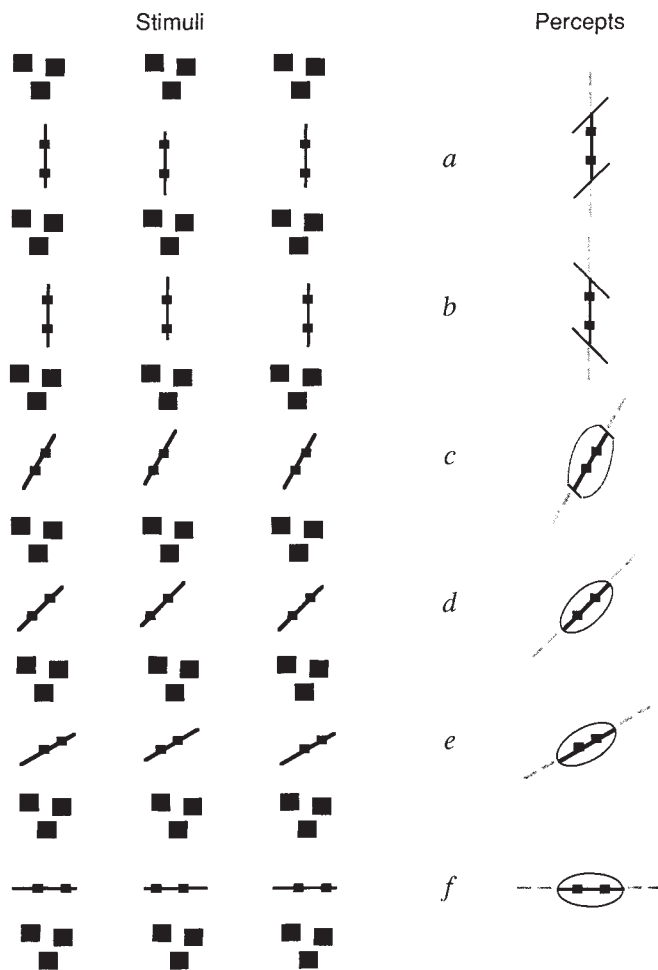


FIG. 3 Patterns demonstrating the rich local structure generated by interpreting the vertical image difference of a single line terminator as a half-occlusion. Cross-fusers should fuse the left two columns, divergers the right two columns. As in the other figures, the description of the subjective contours only holds for line segments which appear behind the small squares in the surround. The far right column depicts the percepts that emerge. The SCs are seen in stereoscopic depth in front of the line segments. The length of the line segment was held constant in the two eyes, but a vertical interocular displacement was added to one of the line segments. The dots in the surround and on the line have only horizontal disparities. In *a*, a single vertical line segment can generate SCs that appear both oriented and in depth. Interchanging the two eye's views leads to a reversal in the perceived orientation of the SCs. To document perceptual sensitivity to this information, four observers were shown 30 stereopairs of patterns (*a* and *b*), presented in random order. Their task was to identify the sign of the SC's slope (that is, positive or negative). No feedback was given to prevent the association of an arbitrary cue to correct identification performance. As can be seen in *g*, observers (*x* axis) were essentially flawless in identifying the sign of the SC's orientation of patterns *a* and *b*. This task could only be performed accurately if the differences in vertical position of the contours were interpreted as half-occlusions. In patterns *c*–*f*, a diagram is presented of the percepts obtained for line segments that deviated from purely vertical. Observers report that the subjective contours not only appear to occlude the ends of the line segments, but also that they tend to 'complete' perceptually and to form illusory windows that surround the line segments. This result reveals strong co-operative interactions between the local SCs formed by interpreting the terminator of a contour as half-occluded. Note that *f* does not contain any vertical displacements, only horizontal ones, yet nonetheless vivid SCs form. This provides a conclusive control experiment that it is not the vertical displacement of the line segments *per se* that is responsible for the formation of the subjective contours; rather, it is the interpretation of the terminators as being half-occluded that generates these illusions.

FIG. 4 Stereopairs used to assess the nature of the interactions between the locally generated SCs revealed in the patterns in Fig. 3a and *b*. Again, cross-fusers should fuse the left two columns, divergers the right two columns. *a*, A stereopair demonstrating that the SCs can combine to form surfaces that are curved within a single, frontoparallel depth plane. *b*, Stereopair demonstrating that these SCs can form coherent contours across non-frontoparallel depth planes. Only the pattern which appears behind the dots in the surround have an occlusion interpretation, and only these figures are stable. The patterns that appear in front of the small squares generate unstable percepts at the terminators of the line segments.

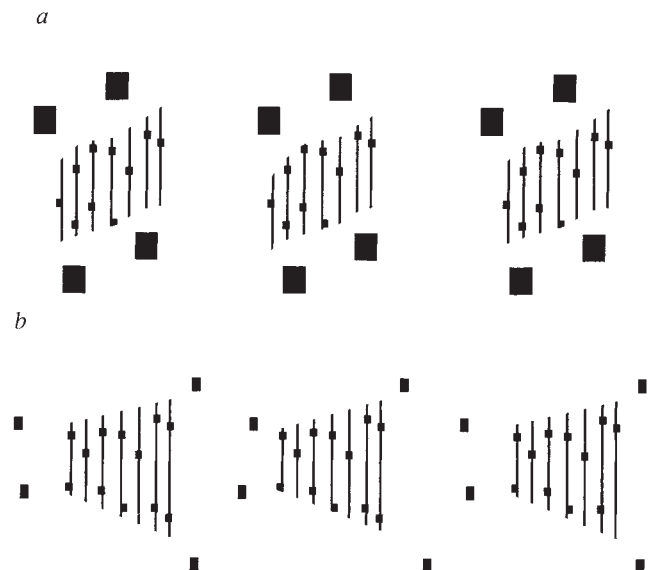


Fig. 2b). This percept was not experienced by any of the 33 observers tested. The second possibility is that portions of the line segments are left unmatched and treated as half-occlusions. An interpretation of this kind should lead to the formation of SCs^{2,11} (Fig. 2c). Compelling SCs are indeed reported by all observers ($n = 33$; $P < 0.001$).

Even for a single contour, the SCs can exhibit both an orientation and a depth. Whereas SCs have been observed previously with half-occlusions that are completely hidden in one eye's view^{2,11}, an oriented depth signal was only perceived when a number of such half-occlusions were aligned. However, for partially occluded, vertically oriented contours, the sign of the occluding contour's orientation (that is, whether it is of positive or negative slope) can be derived by considering where the half-occlusions are relative to the portion of the line that is fused. For example, if the occluding contour has a positive slope (45°, say), it will produce half-occlusions in the right eye if it is above the vertical contour; but if the occluder is below the fused contour, it will produce half-occlusions in the left eye. Thus, it is the geometric relationship between the fused portion of the vertical contour and the half-occlusions which determines the sign of the SC's orientation. Experimental investigation reveals that observers can accurately identify the sign of the SC's orientation for a single vertical line (Fig. 3g). These findings provide conclusive evidence that the vertical displacements of the line segments are being interpreted as half-occlusions.

Another striking property of these SCs is their tendency to interact cooperatively and form global percepts of occluding surfaces. This can be observed for a line segment oriented between zero and ~60° (Fig. 3c-f). Observers report that SCs not only seem to occlude the ends of the line segments, but they also tend perceptually to close and form illusory oval apertures ($n = 33$; $P < 0.001$). This also has a real-world counterpart: an obliquely oriented contour viewed through an aperture would generate some half-occluded features at the ends of the line segments. However, this is not the only interpretation possible: this pattern of stimulation could also have arisen from two disconnected occluding surfaces rather than from a single, closed aperture. The striking quality of this demonstration is the robust tendency for the SCs perceptually to complete and form a single, global contour. Note that it is not the vertical interocular displacement *per se* that is responsible for these illusions, as Fig. 3f contains no vertical displacements. Rather, it is the interpretation of the terminators as half-occlusions that generates the SCs, which can occur for either horizontal or vertical image displacements.

Finally, the stereograms in Fig. 4 reveal that locally generated SCs can perceptually join neighbouring SCs to form curved contours within a single, frontoparallel depth plane (Fig. 4a), or form coherent contours across non-frontoparallel depth planes (Fig. 4b). Hence the interactions between these SCs are not limited to a single depth or co-linear contours. Note also that these SCs are only apparent when the image displacements are consistent with an occlusion configuration. Interchanging the images in the two eyes causes the terminators of the line segments to appear unstable; it does not invert the depth relationships (as would be expected if the terminators were matched and interpreted as disparities).

The relationship between image displacements and occlusion—especially vertical image displacements—has not been appreciated previously. The present findings demonstrate a new way in which vertical image differences can generate percepts of depth, even oriented depth percepts. Although it has long been believed that the primary goal of binocular processing is to generate a disparity map^{5,6,12,13}, the results described here demonstrate that binocular features are actively decomposed into disparities and half-occlusions. The need for this decomposition can be appreciated by considering the information contained in the two kinds of stereoscopic features. Disparity computations provide a rich representation of the surface regions visible to

both eyes, but break down at the discontinuities of object boundaries that occlude other surfaces. The information contained in half-occlusions complements the information contained in disparity signals by providing a rich representation of the depth and orientation of object boundaries that arise at occluding contours. Both forms of information are needed to provide a clear representation of stereoscopic depth in environments replete with occluding contours, and both forms of information are used by the human visual system. □

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Neurotrophin-3 prevents the death of adult central noradrenergic neurons *in vivo*

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NEUROTROPHIN-3 (NT-3)^{1–4} and neurotrophin-4/5 (NT-4)^{5–7}, together with nerve growth factor and brain-derived neurotrophic factor, are members of the neurotrophin family of proteins^{8,9}, which supports the survival of vertebrate neurons. However, no function *in vivo* has been described for NT-4 and limited information is available on the role of the other neurotrophins in the central nervous system *in vivo*. Nerve growth factor prevents the degeneration of lesioned septal cholinergic neurons in the adult brain^{10–13}, whereas brain-derived neurotrophic factor prevents the death of developing motor neurons^{14–16} and a subpopulation of adult septal cholinergic neurons¹⁷. Finally, NT-3 partially prevents the death of facial motor neurons in newborn rats¹⁶. To assess the role of NT-3 and NT-4 in the adult brain *in vivo*, we implanted genetically modified fibroblasts that constitutively express high levels of NT-3 or NT-4. The results show that NT-3, but no other neurotrophin, prevents the degeneration of noradrenergic neurons of the locus coeruleus in a 6-hydroxydopamine lesion model that resembles the pattern of cell loss found in Alzheimer's disease^{18,19}. These results imply that NT-3 may have therapeutic potential for preventing the death of noradrenergic neurons in the locus coeruleus.

Fischer rat 3T3 fibroblasts were co-transfected with a plasmid conferring resistance to the antibiotic G418 and the OVEC expression plasmid¹⁷ containing a rat NT-3 complementary DNA²⁰ or the PCMX expression plasmid containing a rat NT-4 cDNA⁷. Clonal cell lines resistant to G418 were isolated and analysed. Southern blotting showed that cell lines designated F3A-NT3 and F3A-NT4 contained multiple copies of the NT-3 or NT-4 genes, respectively. Northern blotting (data not shown) and ribonuclease protection assays revealed that the cells expressed high levels of the corresponding NT-3 or NT-4 messenger RNA in culture (Fig. 1a, c). In addition, these cell lines continued to express high levels of the introduced neurotrophin gene at least one week after their implantation in the brain, as assessed by both RNase protection assay (Fig. 1a, c) and *in*

situ hybridization (Fig. 1*b, d*). Furthermore, 4 months after the implantation, the F3A-NT3 cell line was still producing at least 10-fold higher levels of NT-3 mRNA than normally found in the dentate gyrus, demonstrating that neurotrophin expression was maintained after prolonged periods in the brain parenchyma.

Conditioned media from the F3A-NT3 or the F3A-NT4 cell lines promoted survival of dissociated E9 chick sensory neurons in a manner indistinguishable from purified recombinant NT-3 or NT-4. Dose-response curves comparing conditioned media and pure neurotrophin revealed that F3A-NT3 cells produced more than 300 ng NT-3 per 10^6 cells per day and that F3A-NT4 cells produced more than 100 ng of NT-4 per 10^6 cells per day. Parental non-transfected Fischer 3T3 cells (designated FR-3T3) expressed very low levels of NT-4 mRNA and did not express detectable levels of NT-3 mRNA. Conditioned media from these cells had no survival-promoting effect on dissociated sensory neurons in culture. A mouse BALB/c 3T3 parental cell line (designated BC-3T3) and a mouse BALB/c 3T3 cell line that constitutively produces high levels of nerve growth factor (NGF) (designated B3E-NGF) were also used as controls. The B3E-NGF cells have previously been shown to stimulate the survival of and fibre outgrowth from fetal cholinergic grafts through the expression of the transfected NGF gene²¹.

The BC-3T3, B3E-NGF, FR-3T3, F3A-NT3 and F3A-NT4 cell lines were unilaterally implanted postero-lateral to the locus coeruleus of adult rats and 30 h later the noradrenergic neurons of this nucleus were selectively lesioned with an ipsilateral injection of 6-hydroxydopamine (6-OHDA) rostral to the locus coeruleus, at the beginning of the dorsal bundle. Seven days after the lesion, the noradrenergic neurons of the locus were

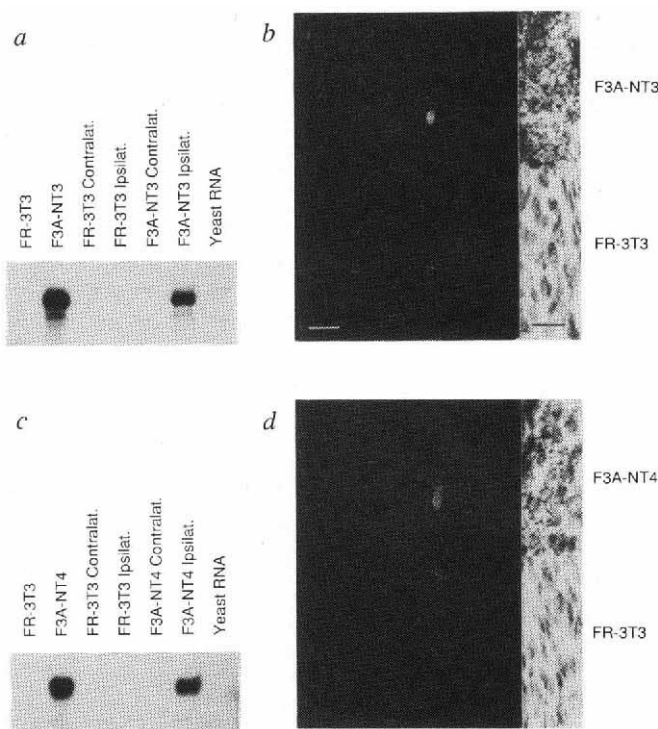


FIG. 1 Expression of NT-3 (*a, b*) and NT-4 mRNAs (*c, d*) by the F3A-NT3 and F3A-NT4 cell lines in culture and one week after implantation in the brain. An RNase protection assay (*a, c*) was performed with specific NT-3 (*a*) and NT-4 (*c*) riboprobes. RNA in lanes FR-3T3, F3A-NT3 and F3A-NT4 was obtained from cells in culture. RNA in lanes labelled 'ipsilat.' was obtained from the area of the brain implanted with the corresponding cell line. As a control for the endogenous levels of neurotrophin in the implantation site, RNA from the same area in the contralateral ('contralat.') non-implanted side was also analysed. Yeast transfer RNA was used as a negative control. For *b* and *d*, *in situ* hybridization was performed with NT-3 (*b*) and NT-4 (*d*) oligonucleotide probes. The upper part of these panels shows brains implanted with the NT-3- (*b*) and NT-4-secreting cell line (*c*), respectively. The lower part of the panels shows the brains implanted with the parental cell line. Insets on the right correspond to bright-field high-power magnification of the implanted fibroblasts. Scale bar in dark-field photograph, 2 mm; in bright-field photomicrographs, 15 µm.

METHODS. To establish the cell lines, a rat NT-3 cDNA⁴ was cloned into the *Pst*I site of the plasmid MRE-(4x)-OVEC (ref. 20). This plasmid, or the PCXM expression vector containing a rat NT-4 cDNA⁷, was linearized and co-transfected with the PBMN-Neo⁸ and the PCH110-lacZ (Pharmacia) plasmids (in a molar ratio 10:1:1) into Fischer rat 3T3 fibroblasts using the calcium phosphate/glycerol technique. Transfected cells were selected with the neomycin analogue G418, isolated, amplified and analysed as described previously²¹. Intracerebral transplantation: cultured fibroblasts collected with Cell Lifters (Costar) were washed twice, counted and resuspended in DMEM without fetal calf serum. Male Fischer 344 rats (200–300 g) were anaesthetized with thiobarbital and stereotactically injected with 0.75×10^6 cells in 3 µl at the following coordinates: AP = -3.1, L = 1.5, DV = -6 (in mm) relative to lambda and the dural surface, with the incisor bar set at -10. The cells typically formed a small focal tumour along the final trajectory of the injection cannula, 500 µm in diameter and 1 mm long, which was located about 150 µm lateral to the caudal end of the locus. One week after the implantation the rats were killed and the brains processed for either RNase protection assay or *in situ* hybridization. RNase protection assays were performed with the RPAII Ribonuclease Protection Assay Kit (Ambion), as described by the manufacturer. NT-3 and NT-4 complementary RNA probes were labelled with [α -³²P]CTP by *in vitro* transcription as described³³. A 3-mm³ block of tissue containing the implanted cell line or, as a control, the same area in the contralateral side of the brain was dissected out using a brain matrix (Activational Systems). The probe was hybridized to 15 µg RNA extracted from three brains in each condition and from the same cell lines in culture. Protected cRNA fragments were separated on 4% polyacrylamide gels under denaturing conditions. *In situ* hybridization was performed on frontal sections (14 µm) obtained from freshly frozen brains. 50- and 48-mer oligonucleotides complementary to rat NT-3 (refs 4, 26, 28, 29) and NT-4 mRNA³³, respectively, were 3'-labelled with [α -³²S]dATP using terminal deoxynucleotidyl transferase (IBI). Hybridization and dipping in Kodak NTB-2 were performed as described^{4, 26, 28, 29, 33}. Dipped slides were counterstained with cresyl violet.

TABLE 1 Mean number of neurons in the dorsal locus coeruleus ($\pm$ s.e.m.) after 6-OHDA lesion and/or implantation of different cell lines

	Cresyl violet		Tyrosine hydroxylase	
	Control side	Operated side	Control side	Operated side
FR-3T3 (<i>n</i> = 3)	672 $\pm$ 22	639 $\pm$ 23	635 $\pm$ 28	668 $\pm$ 29
F3A-NT3 (<i>n</i> = 4)	695 $\pm$ 21	650 $\pm$ 24	687 $\pm$ 24	645 $\pm$ 28
LES (<i>n</i> = 7)	663 $\pm$ 18	387 $\pm$ 21*	634 $\pm$ 20	339 $\pm$ 21*
LES + BC-3T3 (<i>n</i> = 4)	638 $\pm$ 23	358 $\pm$ 21*	622 $\pm$ 24	351 $\pm$ 17*
LES + B3E-NGF (<i>n</i> = 5)	670 $\pm$ 18	430 $\pm$ 37*	670 $\pm$ 20	421 $\pm$ 45*
LES + FR-3T3 (<i>n</i> = 6)	669 $\pm$ 19	385 $\pm$ 25*	663 $\pm$ 23	371 $\pm$ 23*
LES + F3A-NT3 (<i>n</i> = 6)	696 $\pm$ 16	624 $\pm$ 21†	667 $\pm$ 17	608 $\pm$ 27†
LES + F3A-NT4 (<i>n</i> = 6)	626 $\pm$ 5	403 $\pm$ 21*	623 $\pm$ 10	394 $\pm$ 22*

6-Hydroxydopamine lesions (LES) and the indicated cell-line implants were performed in adult male Fischer 344 rats (*n* = 41) as described in legends to Figs 1 and 2. Seven days after the last surgery, the number of noradrenergic neurons, visualized by cresyl violet or TH immunohistochemistry, was assessed in serial sections every 98 µm along the complete dorsal segment of the locus coeruleus of both the operated and the control side. Quantification of the brain damage was by direct visual counting of intact neurons using a Nikon microphot-FXA microscope. In TH-immunostained sections, neurons showing a clearly stained cytoplasm were counted as positive in blind duplicate or triplicate determinations. In cresyl violet-stained sections, neurons (identified by the presence of a typical nucleus with a clear nucleoplasm and a prominent nucleolus) that showed the morphological features of the noradrenergic neurons of the locus coeruleus (fusiform or multipolar pigmented cytoplasm) were also counted as positive in blind duplicate or triplicate determinations at a magnification of $\times 100$. Positive neuron counts were taken for statistical analysis and were not corrected for split nucleoli as described⁶.

* $P < 0.005$, compared with the control side; † $P < 0.005$, compared with the operated side of animals with lesion alone or lesion and FR-3T3 implant, using Student's *t*-test, two-tailed.

FIG. 2 Pattern of cell loss in the 6-hydroxydopamine-lesioned locus coeruleus. The number of tyrosine hydroxylase-positive neurons in the lesioned side (●) is compared with the non-lesioned side (□). Values represent mean $\pm$ s.e.m. * $P < 0.01$ compared with the number in the lesioned side using Student's *t*-test, two-tailed.

METHODS. Male Fischer 344 rats (200–300 g; $n = 6$) were anaesthetized with thiobarbital, treated with 4 mg kg⁻¹ amfonelic acid (intraperitoneally, i.p.) and stereotactically injected with 8 μ g 6-OHDA (Sigma) at the following coordinates: AP=1.5, L=1.2, DV=-7.1 (in mm) relative to lambda and the dural surface, with the incisor bar set at 5 mm over zero; that is, 100 μ m lateral and inferior to the rostral end of the locus. Seven days after the 6-OHDA lesion the animals were killed by an ether overdose and perfused transcardially with 4% paraformaldehyde. The brains were postfixed overnight at 4 °C, embedded in 10% sucrose for 2 days and serial cryostat sections (14 μ m) were prepared from the entire locus coeruleus. Sections every 98 μ m were processed for TH immunohistochemistry using a mouse monoclonal anti-rat TH antibody (Incstar) and a lissamine-rhodamine donkey anti-mouse IgG (Jackson) according to the manufacturer's recommendations. Cells were counted as described for Table 1.

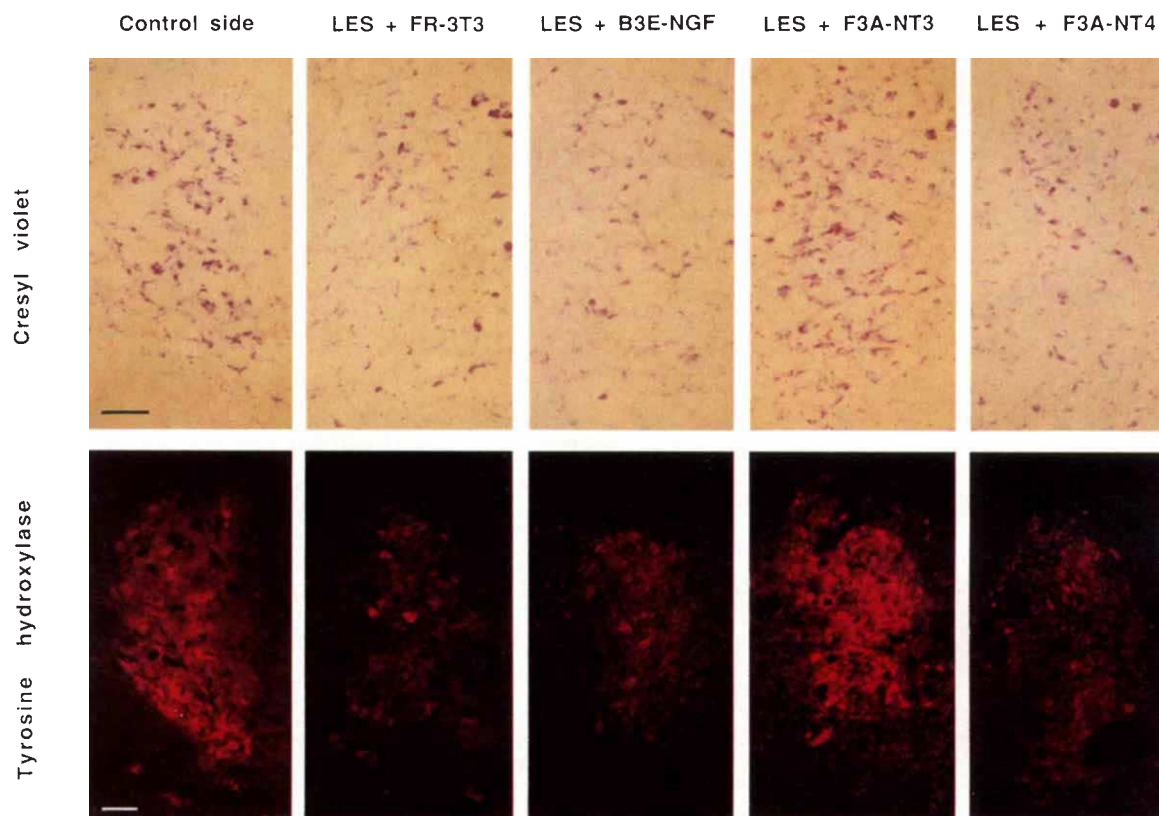
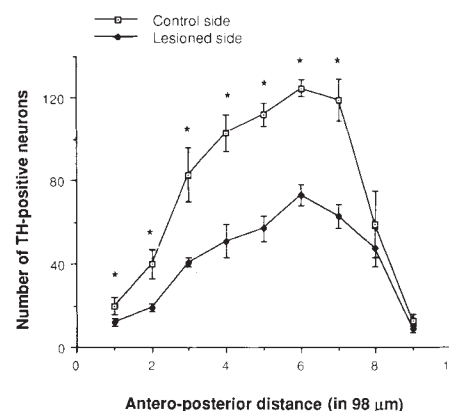


FIG. 3 Photomicrographs of tyrosine-hydroxylase- and cresyl-violet-stained neurons of the anterior part of the locus coeruleus in the control side, compared with the 6-OHDA-lesioned side (LES) implanted with the FR-3T3, B3E-NGF, F3A-NT3 or F3A-NT4 cell lines. Scale bars, 100 μ m. Control sides show the contralateral side of the 6-OHDA-lesioned side implanted with the F3A-NT3 cell line.

METHODS. The Fischer rat 3A cell lines producing NT-3 (F3A-NT3) or NT-4 (F3A-NT4) and the BALB/c 3E cell line producing NGF²¹ (B3E-NGF) were grown in DMEM, 10% fetal calf serum, 1% penicillin-streptomycin, 1% glutamine and 200 μ g ml⁻¹ G418. The parental cell lines (FR-3T3 and BC-3T3) were grown in media without G418. The different cell lines were collected and stereotactically implanted in male Fischer 344 rats (200–300 g) ($n = 34$) as described for Fig. 1. The location chosen for the injection preserved the integrity of the nucleus and allowed access of the proteins secreted by the cell lines to the neurons of the locus

coeruleus. Animals implanted with the BALB/c cell lines were daily immunosuppressed with cyclosporin (10 mg kg⁻¹, i.p.). Thirty hours after the implant, the rats were re-anaesthetized and lesioned as described for Fig. 2. Seven days later they were killed, serial cryostat sections (14 μ m) were prepared from the entire locus coeruleus and the correct positions of the implant and lesion sites were confirmed. Sections every 98 μ m were stained with cresyl violet and the cell bodies of neurons with morphological features of noradrenergic neurons of the locus coeruleus (fusiform or multipolar pigmented cytoplasm) were counted at high magnification. TH-positive cell bodies were counted in adjacent sections and processed for TH immunohistochemistry as described for Fig. 2. The number of TH-positive neurons was not statistically different from the number of pigmented neurons stained by cresyl violet in any experimental condition.

visualized using antibodies to tyrosine hydroxylase (TH), a marker for catecholaminergic neurons, and by Nissl staining. Survival was determined by counting, in adjacent serial sections, the numbers of multipolar and bipolar pigmented neurons stained with cresyl violet and the numbers of TH-positive neurons in the ipsilateral and contralateral sides.

In animals with no cells implanted the 6-OHDA injection caused a 45% loss of noradrenergic neurons compared with the control side in the same animal. The cell loss was more severe in the anterior than the posterior part of the nucleus (Fig. 2). The numbers of TH-positive and cresyl-violet stained neurons decreased to the same extent (Table 1), indicating that the decrease in the number of TH-positive neurons was due to a loss of catecholaminergic neurons and not to a decrease of TH levels below the detection limit. In non-lesioned animals implanted unilaterally with either parental or neurotrophin-producing cell lines, the numbers of TH-positive and cresyl-violet stained neurons did not change compared with the control side (Table 1). In the 6-OHDA-lesioned animals, the BC-3T3 or FR-3T3 parental cells did not prevent the decrease in the number of TH-positive neurons or cresyl-violet-positive neurons on the side ipsilateral to the lesion (Fig. 2 and Table 1), showing that the parental fibroblasts *per se* had no effect on the survival of noradrenergic neurons in the locus coeruleus.

The ipsilateral implantation of the B3E-NGF or F3A-NT4 cell lines in 6-OHDA-lesioned animals had no significant effect on the numbers of TH-positive and cresyl-violet-positive neurons compared with lesioned animals without implants or implanted with the respective parental fibroblasts (Fig. 3 and Table 1). These results show that neither NGF nor NT-4 promotes the survival of the noradrenergic neurons of the locus coeruleus. In addition, preliminary data obtained with a Fischer 3T3 cell line, which produced more than 100 ng of brain-derived neurotrophic factor (BDNF) per 10^6 cells per day, indicates that BDNF is also unable to support the survival of these neurons after 6-OHDA lesions (E.A. and I. Neveu, unpublished results). In contrast, implantation of the NT-3-producing F3A-NT3 cells significantly reduced the loss of noradrenergic neurons in the ipsilateral locus coeruleus (Fig. 3 and Table 1). In all six animals analysed, 80% of the 6-OHDA-lesioned noradrenergic neurons were rescued from degeneration by the F3A-NT3 implants (Fig. 3 and Table 1). Both cresyl-violet- and TH-positive neurons were rescued to the same extent, indicating that the implant prevented the degeneration of noradrenergic neurons in the locus coeruleus. The fact that this was not observed with any other cell line shows that the effect was elicited by NT-3, produced by the F3A-NT3 cells.

It has been shown previously that both NT-3 and NT-4²² support the survival of embryonic noradrenergic neurons of the cultured locus coeruleus. However, the present results show that NT-3 specifically promotes the survival of adult locus coeruleus neurons *in vivo*, as neither NGF nor BDNF or NT-4 prevented 6-OHDA-induced cell death. These findings are consistent with the finding that neurons of the locus coeruleus in the adult do not express any *trk* receptor other than *trkC* mRNA²³, encoding an essential component of the high-affinity NT-3 receptor²⁴. These results together suggest that the different sensitivity and responsiveness to NT-4 of embryonic neurons in culture compared with adult neurons *in vivo* may be due to a possible transient expression of *trkB* mRNA during development.

The distribution of the high-affinity NT-3-binding sites²⁵ and NT-3 mRNA^{1-4,26-29} in the central nervous system correlates well with the main projection fields of the noradrenergic neurons of the locus coeruleus. This is consistent with a target-derived neurotrophic role of NT-3 for central noradrenergic neurons. The diffuse distribution of the projections from the locus coeruleus involve this nucleus in the regulation of many brain functions such as attention and memory³⁰. Furthermore, in Alzheimer's-type dementia there is a marked reduction in the noradrenergic innervation of the target-fields and in the number

of noradrenergic neurons, mainly in the anterior part of the locus coeruleus^{18,19}. The lesion model used in the present study resembles Alzheimer's disease both in the pattern of cell loss in the locus (compare Fig. 1 in this study with Fig. 3 of German *et al.*¹⁹) and in the proposed mechanism of cell death, oxidative stress^{31,32}. The finding that NT-3 is a survival-promoting factor for central noradrenergic neurons in this lesion model suggests that NT-3 may also prevent the death of noradrenergic neurons in neurodegenerative disorders affecting the locus coeruleus, such as Alzheimer's disease. □

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A crucial role for neurotrophin-3 in oligodendrocyte development

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THE neurotrophins nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5 promote the survival of subpopulations of vertebrate neurons *in vitro*, but so far only nerve growth factor has been demonstrated to be essential for

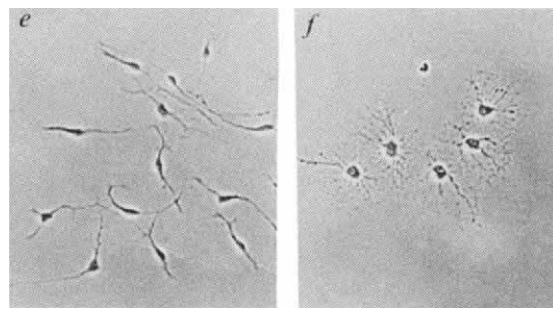
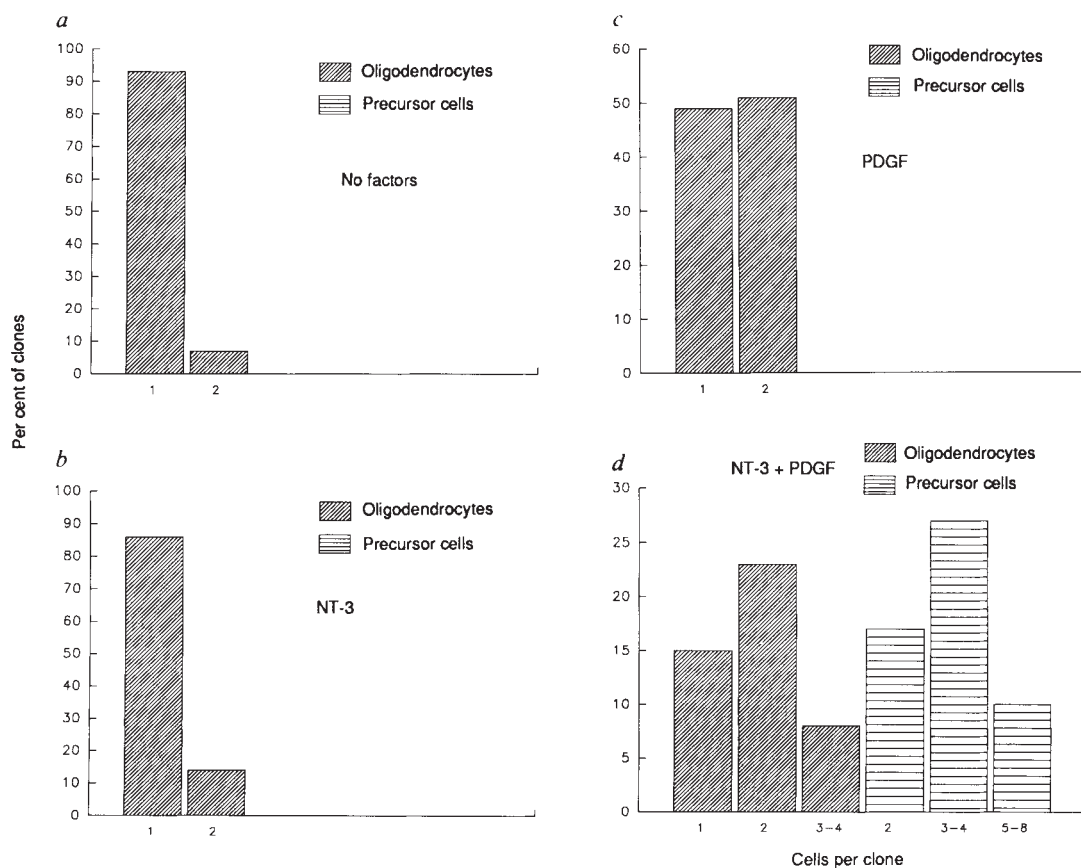
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normal neuronal development¹⁻⁴; no neurotrophin has previously been shown to function in normal glial cell development. We found recently that neurotrophin-3 promotes the survival of pure oligodendrocyte precursor cells *in vitro*, and, although by itself it induces only a small percentage of these cells to synthesize DNA, in combination with platelet-derived growth factor it induces the majority of them to do so⁵. Neither of these factors, however, has been shown to contribute to oligodendrocyte precursor cell proliferation *in vivo* or to stimulate pure populations of these cells to proliferate (as opposed to synthesize DNA) *in vitro*. Here we show that neurotrophin-3 and platelet-derived growth factor collaborate to promote clonal expansion of oligodendrocyte precursor cells *in vitro* and to drive the intrinsic clock that times oligodendrocyte development⁶. We also show that neurotrophin-3 helps stimulate the proliferation of oligodendrocyte precursor cells *in vivo* and is thus required for normal oligodendrocyte development.

When purified oligodendrocyte precursor cells (also termed O-2A progenitor cells) isolated from postnatal day 1 rat optic nerves were cultured at clonal density in serum-free medium containing insulin ($5 \mu\text{g ml}^{-1}$), all of the cells prematurely differentiated into oligodendrocytes during the first 4 days of culture (Fig. 1a), as expected in the absence of mitogens^{7,8}. Surprisingly, when either neurotrophin-3 (NT-3) or platelet-derived growth factor (PDGF) was added alone at plateau concentrations⁵ to the culture medium, all of the cells also differentiated into oligodendrocytes after 4 days of culture, although some of the cells divided once before differentiating (Fig. 1b, c). Clonal expansion did occur, however, when the cells were cultured in NT-3 and PDGF together: most of the cells now divided more than once over 4 days of culture and the cells in more than half of the clones were still precursors rather than oligodendrocytes (Fig. 1d-f). Some clones were still proliferating after 8 days, with the

FIG. 1 The proliferative capacity of oligodendrocyte precursor cells cultured for 4 days at clonal density in serum-free medium containing a high concentration of insulin. Note that all of the cells differentiated into oligodendrocytes (cross-hatched) except in cultures containing both NT-3 and PDGF (d). After 4 days of culture, the average size of oligodendrocyte precursor cell clones was 4.1 ± 0.3 cells per clone, but was 0 cells per clone in insulin (a), NT-3 (b) or PDGF (c) alone. At least 100 clones were scored for each condition. The experiment was repeated three times with nearly identical results. e, An oligodendrocyte precursor cell clone; and f, an oligodendrocyte clone in cultures treated with NT-3 and PDGF. Note that oligodendrocyte precursor cells have a bipolar morphology, while oligodendrocytes have multiple interconnecting processes⁶. One of the cells in f is dead. Scale bar, $50 \mu\text{m}$.

METHODS. Oligodendrocyte precursor cells from optic nerves taken from rats at postnatal day 1 were purified to >99.95% homogeneity by sequential immunopanning as described previously⁹, and the cells were cultured at a clonal density in PDL-coated tissue culture dishes (60 mm, Falcon) in 2.5 ml of serum-free Dulbecco's modified Eagle's medium (DMEM) containing bovine insulin ($5 \mu\text{g ml}^{-1}$), human transferrin ($100 \mu\text{g ml}^{-1}$), bovine serum albumin ($100 \mu\text{g ml}^{-1}$), progesterone (60 ng ml^{-1}), putrescine ($16 \mu\text{g ml}^{-1}$), sodium selenite (40 ng ml^{-1}), thyroxine (40 ng ml^{-1}), triiodothyronine (30 ng ml^{-1}) and, where indicated, various growth factors. Recombinant human PDGF-AA (Peprotech) was used at 10 ng ml^{-1} . Recombinant mouse NT-3 was prepared as described previously²⁰ and used at 5 ng ml^{-1} . Both of these concentrations were well onto the plateau of the dose-response curves⁵. Postnatal day 1 rates were used to maximize the proliferative potential of the precursor cells (our unpublished observations). The numbers of cells cultured were titrated to achieve about 50 clones per dish. The cultures were fed with fresh medium and growth factors (50% volume replacement) every 3 days. After 4 days of culture the number of cells per clone and their identity were tabulated. Cells were identified as either



oligodendrocytes or precursor cells by their characteristic morphologies, as described previously⁶. The absence of clones containing a single precursor cell in d suggests that there was little net migration between clones. For e and f, purified oligodendrocyte precursor cells were cultured for 6 days at clonal density in both PDGF-AA and NT-3 as above and were photographed in an inverted phase-contrast microscope.

largest clones containing 60 cells (not shown). Clonal expansion did not occur when NT-3 was replaced with nerve growth factor (NGF, 50 ng ml⁻¹; Boehringer-Mannheim) or brain-derived neurotrophic factor (BDNF; 50 ng ml⁻¹). Surprisingly, the presence of insulin-like growth factor-1 (IGF-1, 20 ng ml⁻¹) or high concentrations of insulin (5 µg ml⁻¹) was necessary for clonal expansion of postnatal day 8 but not postnatal day 1 oligodendrocyte precursor cells (not shown).

In the presence of NT-3 and PDGF, oligodendrocyte differentiation occurred synchronously within a clone, indicating that the intrinsic clock thought to limit the maximal number of divisions⁶ operates under these conditions. The oligodendrocytes that developed in the presence of NT-3 and PDGF died within several days⁹ unless ciliary neurotrophic factor (CNTF) was also added to the medium to promote their survival^{5,10}. In the presence of PDGF, NT-3 and CNTF, oligodendrocyte clones as large as 130 cells were observed after 10–12 days in culture (not shown).

In previous experiments, clonal expansion of oligodendrocyte precursor cells was achieved by culturing individual cells in microwells in medium continuously conditioned by cortical astrocytes^{6,8}, which are known to secrete PDGF^{11,12}. This finding, taken together with the results shown in Fig. 1, suggested that cultured astrocytes might secrete NT-3. To check this, we tested the ability of a neutralizing monoclonal antibody to NT-3 to inhibit DNA synthesis in oligodendrocyte precursor cells in mixed optic nerve cell cultures. As shown in Fig. 2, the anti-NT-3 antibody caused an approximately 50% reduction in the number of oligodendrocyte lineage cells that incorporated bromodeoxyuridine (BrdU) into DNA, indicating that optic nerve cells secrete NT-3 in culture. The control antibody had no effect. Similarly, highly purified optic nerve type-1 astrocytes stimu-

lated the precursor cells to incorporate BrdU into DNA; the addition of anti-NT-3 antibody decreased this incorporation by about 50% and this inhibition was reversed by the addition of excess amounts of recombinant NT-3 (Fig. 2c). Thus, optic nerve astrocytes secrete NT-3, at least during their first few days in culture. It is likely that these cells also synthesize NT-3 *in vivo*, as NT-3 messenger RNA has recently been detected in the developing rat optic nerve¹³.

To determine whether NT-3 also stimulated astrocytes themselves to synthesize DNA, we studied the effect of NT-3 and anti-NT-3 antibody on the purified type-1 astrocytes. Unlike purified oligodendrocyte precursor cells, the purified astrocytes synthesized DNA in the absence of added mitogens, but neither NT-3 nor anti-NT-3 antibody significantly affected this basal level of DNA synthesis (not shown), suggesting that NT-3 does not influence DNA synthesis in type-1 astrocytes.

To determine whether oligodendrocyte lineage cells express the *trkC* gene, which encodes the NT-3 receptor TrkC, a frag-

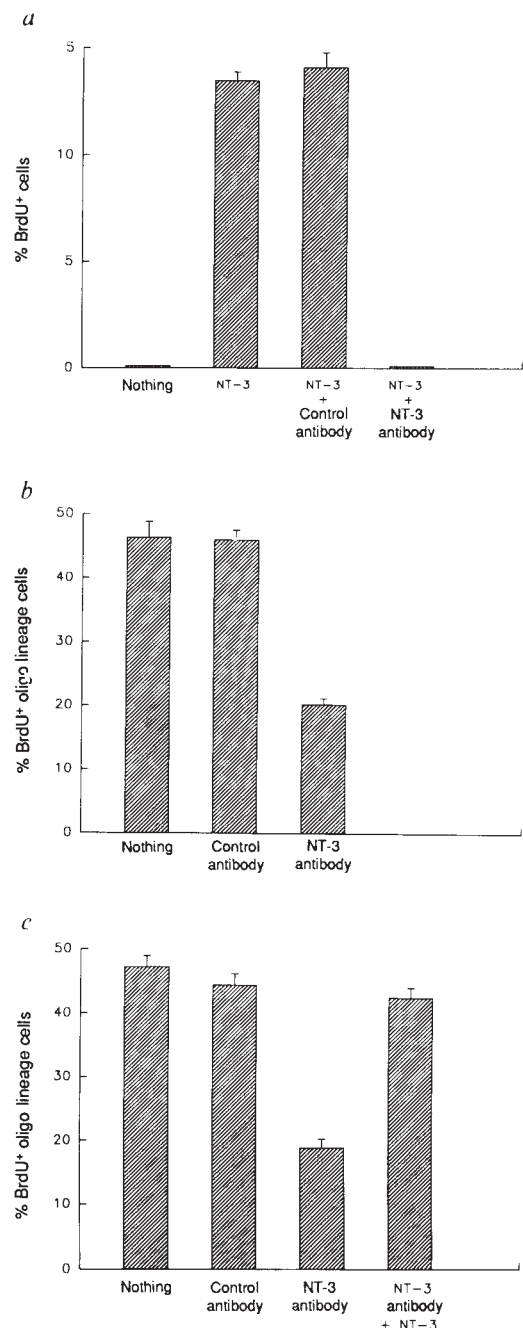


FIG. 2 The effect of a neutralizing anti-NT-3 antibody on the incorporation of BrdU into DNA by purified oligodendrocyte precursor cells (a), oligodendrocyte precursor cells in mixed optic nerve cultures (b) and oligodendrocyte precursor cells in cocultures of purified precursor cells and optic nerve type-1 astrocytes (c).

METHODS. a, Purified postnatal day 8 oligodendrocyte precursor cells were cultured in the presence or absence of NT-3 (0.2 ng ml⁻¹) for 36 h. An IgG1 monoclonal anti-NT-3 antibody, which specifically neutralizes the activity of NT-3 (see below), was added as serum-free hybridoma supernatant (50:50) to some cultures. An IgG1 monoclonal anti-BrdU antibody²¹ was used as a control. Over the last 12 h of culture, BrdU (Boehringer-Mannheim; 10 µM) was added to all cultures (note that the control anti-BrdU antibody does not bind to BrdU that has not been incorporated into DNA). b, Postnatal day 2 optic nerve cell suspensions were prepared with trypsin as described previously²² and cultured for 48 h in serum-free medium (described in Fig. 1 legend) containing a small amount of PDGF (1 ng ml⁻¹) to prevent premature differentiation of the oligodendrocyte precursor cells. To determine the percentage of oligodendrocyte lineage cells that had incorporated BrdU, the cultures were labelled with three monoclonal antibodies—an IgM A2B5 antibody²³ to detect oligodendrocyte precursor cells, an IgG3 anti-galactocerebroside (GC) antibody²⁴, to detect oligodendrocytes, and an IgG2a anti-BrdU antibody²⁵, as described previously⁹. c, Optic nerve type-1 astrocytes were isolated from postnatal day 2 optic nerve cell suspensions to >99% purity by a sequential immunopanning procedure similar to that described previously for oligodendrocyte precursor cell purification⁹. The cultures were treated identically to those described in a, except that in some wells NT-3 (200 ng ml⁻¹) was added in addition to anti-NT-3 antibody. All results are shown as means ± s.e.m. (n = 3). In neuron-survival assays, the anti-NT-3 monoclonal antibody (designated mAb 12) completely inhibited the activity of NT-3, but not those of NGF, BDNF or *Xenopus* NT-4 (F.G. et al., manuscript in preparation). Control experiments demonstrated that conditioned medium from the hybridoma cells producing the anti-NT-3 antibody completely neutralized the ability of NT-3 to stimulate BrdU incorporation into purified oligodendrocyte precursor cells *in vitro*, whereas conditioned medium from hybridoma cells secreting anti-BrdU antibody had no effect (a).

ment of rat *trkC* (base pairs (bp) 1,990–2,241)¹⁴ was amplified from the total RNA¹⁵ of purified postnatal day 8 oligodendrocytes and their precursors by the reverse-transcription polymerase chain reaction¹⁶. Both amplicates contained a predominant fragment of 250 bp, about the same size as a full-length *trkC* complementary DNA¹⁴. To characterize the *trkC* bands further, the material was reamplified and directly sequenced; this confirmed the presence of the insert-less, full-length *trkC* (not shown).

Oligodendrocyte precursor cells in the neonatal optic nerve are rapidly dividing: about 17% of them have incorporated BrdU into DNA 1 h after a single intraperitoneal injection of

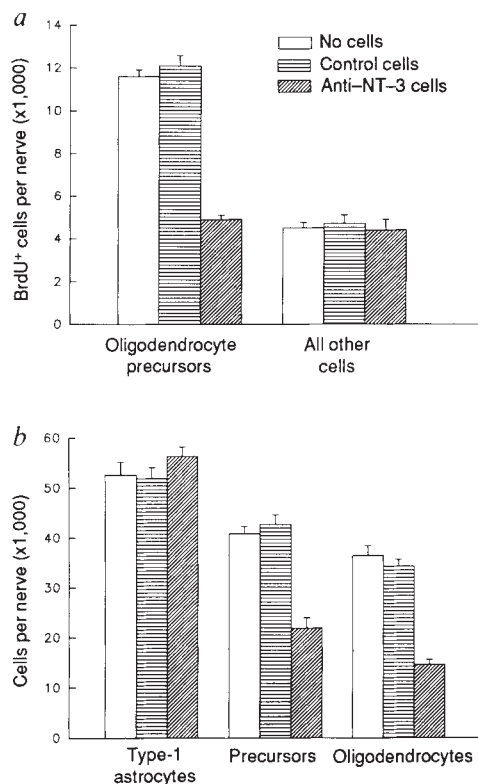


FIG. 3 The effect of anti-NT-3 antibody on cell proliferation (a) and cell number (b) in the developing rat optic nerve.

METHOD. Mouse hybridoma cells secreting either the neutralizing IgG1, anti-NT-3 monoclonal antibody, or an irrelevant IgG1, anti-BrdU antibody, were injected into the subarachnoid space of postnatal day 1 rats, when the first oligodendrocytes begin to appear²⁶. One million cells were slowly injected under ether anaesthesia through the right frontal skull using a 10- μ l Hamilton syringe. Both IgG and IgM antibodies can be rapidly delivered into the optic nerves of developing postnatal animals using this procedure^{9,27}. Six days after the injection, the animals were killed and the optic nerves were removed and examined. Values represented means $\pm$ s.e.m. ($n = 4$). The total numbers of cells in normal, control and test nerves were determined by measuring the DNA content as described previously⁹. The number of BrdU⁺ oligodendrocyte precursor cells and the percentages of cells of each type per nerve were determined by dissociating the cells and staining them by immunofluorescence with cell-type-specific antibodies against astrocytes, oligodendrocyte precursor cells and oligodendrocytes, as described previously⁹. The immunofluorescence values (per cent) were multiplied by the total number of cells per nerve (determined by DNA measurement) to give the number of each cell type per nerve. The decrease in the number of oligodendrocyte lineage cells in the anti-NT-3-antibody-treated animals is unlikely to be an indirect effect of the antibody on the number of retinal ganglion cells: NT-3 does not promote the survival of purified retinal ganglion cells²⁸ in culture (our unpublished observations) and immunolabelling retinal cell suspensions with the Thy1.1 antibody (to identify ganglion cells²⁹) demonstrated that the number of retinal ganglion cells was not decreased by the anti-NT-3 antibody treatment (not shown).

BrdU (Table 1). Whereas single mitogens or a combination of PDGF and basic fibroblast growth factor (bFGF) were unable to induce a comparable rate of BrdU incorporation in purified postnatal day 1 oligodendrocyte precursor cells *in vitro*, the combination of NT-3 and PDGF did (Table 1). Surprisingly, the further addition of bFGF to the combination of NT-3 and PDGF decreased BrdU incorporation almost threefold (Table 1). These findings are consistent with the possibility that NT-3 and PDGF collaborate to promote oligodendrocyte precursor cell proliferation *in vivo*.

To test this possibility, we delivered the neutralizing monoclonal anti-NT-3 antibody into developing rat optic nerve for 6 days, beginning at postnatal day 1, by injecting anti-NT-3-secreting hybridoma cells into the subarachnoid space of rats on postnatal day 1. The proportion of oligodendrocyte precursor cells that incorporated BrdU after a single intraperitoneal injection of BrdU was decreased by more than 50% in animals that had received the anti-NT-3 antibody-secreting hybridoma cells, but was unaffected in the animals that received control hybridoma cells (Fig. 3a). Moreover, the nerves from the anti-NT-3-antibody-treated animals were visibly decreased in diameter compared with control nerves, and this difference was especially striking when older animals (in which the rate of oligodendrocyte generation is higher) were treated. The total number of cells per nerve, determined by measuring the total amount of DNA, was decreased by 18% in the anti-NT-3-antibody-treated animals compared with normal animals or animals that had received control hybridoma cells (not shown). Whereas the number of astrocytes was not changed by anti-NT-3 antibody delivery, the numbers of oligodendrocyte precursor cells and oligodendrocytes were each approximately halved (Fig. 3b).

These results demonstrate that NT-3 is required for the normal development of oligodendrocytes in the rat optic nerve. Although astrocytes are probably one source of NT-3 in the developing optic nerve, we cannot exclude the possibility that retinal ganglion cell axons are another. Neurotrophins are best known for their effects on developing vertebrate neurons (for review see ref. 17). Although NT-3 is the only growth factor

TABLE 1 The effect of growth factors on BrdU incorporation by oligodendrocyte precursor cells

	Factors added	%BrdU ⁺ cells
<i>In vitro</i>	None	0
	PDGF	9.1 $\pm$ 0.3
	NT-3	3.2 $\pm$ 0.2
	bFGF	6.8 $\pm$ 0.7
	PDGF and bFGF	5.5 $\pm$ 0.7
	PDGF and NT-3	17.4 $\pm$ 0.4
	PDGF, NT-3 and bFGF	6.3 $\pm$ 0.4
<i>In vivo</i>	None	16.6 $\pm$ 0.4

Approximately 5,000 oligodendrocyte precursor cells, purified as described for Fig. 1, were cultured in poly-D-lysine (PDL)-coated wells of a 96-well Falcon culture dish in 100 μ l of serum-free medium containing insulin (5 μ g ml⁻¹) and plateau concentrations of the recombinant growth factors indicated. PDGF (the AA homodimer form) was used at 10 ng ml⁻¹, NT-3 at 10 ng ml⁻¹ and bFGF (Peprotech, New Jersey) at 10 ng ml⁻¹. After 36 h, BrdU (10 μ M) was added, and 1 h later the cells were fixed and labelled with monoclonal anti-BrdU antibody as described previously⁹. To determine the amount of DNA synthesis occurring in oligodendrocyte precursor cells *in vivo* at postnatal day 1, cells in S phase were labelled *in vivo* by an intraperitoneal injection of BrdU (0.1 mg per g body weight). The animals were killed 60 min later and optic nerve cell suspensions were prepared as described previously⁹. Cells were cultured on PDL-coated glass coverslips for several hours before fixation with 4% paraformaldehyde for 90 s at room temperature, then double-labelled by indirect immunofluorescence with monoclonal anti-BrdU and A2B5 antibodies, as described previously⁹. Oligodendrocyte precursor cells were identified by their characteristic morphology and by the presence of A2B5. The results shown are means $\pm$ s.e.m. of three cultures.

demonstrated to play a part in stimulating oligodendrocyte precursor cell proliferation *in vivo*, it seems likely that PDGF and IGFs also play important parts, as they do *in vitro*. Although the combination of PDGF and bFGF has been reported to support the proliferation of oligodendrocyte precursor cells *in vitro*¹⁸, in our cultures it did so much less effectively than the combination of NT-3 and PDGF. As oligodendrocyte differentiation *in vitro* is profoundly inhibited in the presence of PDGF and bFGF^{18,19}, even in the presence of NT-3 (our unpublished observations), it seems probable that, unlike NT-3, bFGF is not available to most precursor cells in the optic nerve during the period of oligodendrocyte development. We conclude that, in culture at least, multiple growth factors are required for the proliferation of oligodendrocyte precursor cells, just as multiple growth factors are required for the long-term survival of oligodendrocytes⁹. □

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A mutation in the *RET* proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma

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MULTIPLE endocrine neoplasia type 2 (MEN 2) comprises three clinically distinct, dominantly inherited cancer syndromes. MEN 2A patients develop medullary thyroid carcinoma (MTC) and pheochromocytoma. MEN 2B patients show in addition ganglioneuromas of the gastrointestinal tract and skeletal abnormalities. In familial MTC, only the thyroid is affected. Germ-line mutations of the *RET* proto-oncogene have recently been reported in association with MEN 2A and familial MTC^{1,2}. All mutations occurred within codons specifying cysteine residues in the transition point between the *RET* protein extracellular and transmembrane domains. We now show that MEN 2B is also associated with mutation of the *RET* proto-oncogene. A mutation in codon 664, causing the substitution of a threonine for a methionine in the tyrosine kinase domain of the protein, was found in all nine unrelated MEN 2B patients studied. The same mutation was found in six out of 18 sporadic tumours.

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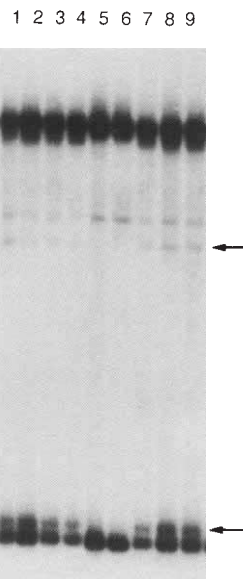


FIG. 1 Single-strand conformational polymorphism (SSCP) analysis¹⁴ of *RET*. The autoradiogram shows SSCP patterns of PCR-amplified products of exon 16 of the *RET* gene. MEN 2B patients are indicated as 1–4 and 7–9, the parents of patient 4 are indicated as 5 and 6. Arrows indicate the positions of the SSCP variant bands.

METHODS. The PCR primers designed were rRET 16 (5'-AGGGATAGGG-CCTGGGCTTC-3') and rRET 16 (5'-TAACCTCCACCCCAAGAGAG-3'). They give a PCR product of 192 base pairs (bp) containing the entire exon 16 and part of the flanking intronic sequences. Radioactive PCR amplification was carried out on 150 ng of DNA in a total volume of 30 µl for 25 cycles at 92 °C for 35 s, 58 °C for 35 s and 72 °C for 60 s using [α -³²P]dCTP. SSCP analysis was carried out under three different sets of conditions. The variant pattern could be detected in all. The figure shows an autoradiogram of a 6% acrylamide gel containing 10% glycerol. DNA was electrophoresed in 45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA, pH 8.0, at 20 °C.

As the MEN 2 syndromes resemble several other hereditary cancers in occurring both in a familial form, characterized by a dominant pattern of inheritance, and in a sporadic form, involvement of tumour-suppressor genes seemed likely³. The disease locus for both MEN 2A and MEN 2B has been assigned to chromosome 10 (refs 4–6). Still, MTCs and pheochromocytomas seldom show allelic losses for this chromosome, although

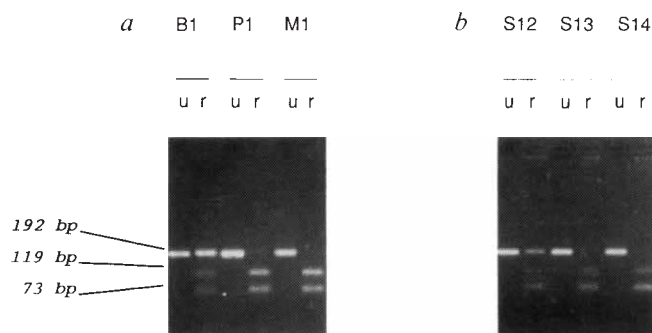


FIG. 2 Restriction patterns of PCR products of exon 16 with *FokI*. The described mutation eliminates a *FokI* restriction site. Whereas normally both alleles are restricted, in the presence of the mutation the mutated allele will not be restricted. *a, b*, The undigested (*u*) and restricted (*r*) PCR products of constitutive DNA from a MEN 2B patient, designated B1, and from his parents designated, P1 and M1 (*a*), and tumour DNA from three sporadic MTC patients, S12-S14. As can be seen, S12 has the mutation whereas S13 and S14 do not (*b*).

METHODS. A nonradioactive PCR was carried out as described in Fig. 1 legend. The PCR products were purified in low-melting-point agarose, isolated (Sephaglas BandPrep kit; Pharmacia) and digested for 1 h using 2 U *FokI* (Boehringer) in the restriction buffer recommended by the manufacturer. The samples were run in a 1% normal agarose/2% low-melting-point agarose gel.

these would be expected in the case of a tumour-suppressor mechanism^{7,8}. Recently, mutations in the *RET* proto-oncogene were described in association with MEN 2A^{1,2}. If these mutations underlie the MEN 2A phenotype, a dominant or dominant-negative mechanism is a more probable explanation for this syndrome. Thus far, no mutations have been reported to be associated with MEN 2B.

Determination of the gene structure of *RET*⁹ allowed us to design specific intronic primer pairs for almost all exons. When these were used in a single-strand conformational polymorphism (SSCP) analysis on constitutive DNA from MEN 2B patients, a variant pattern for exon 16 was found in the DNA from all nine MEN 2B patients, but not in DNA from 70 independent persons, nor in DNA available from the parents of three of the MEN 2B patients (Fig. 1). Sequence analysis of the polymerase chain reaction (PCR) products of exon 16 revealed a T→C transition at position 2,684 (ref. 10) in one of the alleles of all the MEN 2B patients. A threonine (ACG) is thereby substituted for a methionine (ATG) at codon 664. As the mutation eliminates a *FokI* restriction site (GGATG(N)9/13→GGACG(N)9/13), digestion of the PCR products of exon 16 by this restriction enzyme was used to confirm the presence of the mutation in all MEN 2B patients (Fig. 2). As the MEN 2B patients analysed originated from The Netherlands, Italy and North America, our results suggest that a single *RET* mutation may underlie most, if not all, of the MEN 2B cases.

When analysing tumour DNA from 18 sporadic MTC patients for this mutation, we detected in six cases the same SSCP variant, *FokI* restriction pattern and sequence as found in the MEN 2B patients. No indication for an exon 16 mutation was obtained for five sporadic pheochromocytomas. The same held true for 15 independent MEN 2A patients.

The codon 664 mutation affects the intracellular tyrosine kinase domain of *RET*¹⁰, whereas the mutations associated with MEN 2A are all in the extracellular region, close to the transmembrane domain. This may account for the different phenotypes of MEN 2A and MEN 2B. Occurrence of a somatic

mutation of the *RET* proto-oncogene may lead to neoplasia of the tissue involved. This explains the finding in sporadic MTC of mutations affecting the same codons as in MEN 2A² and MEN 2B (the present study). No constitutive DNA was available for our patients with sporadic MTC. However, the absence of additional MEN 2B symptoms makes a germ-line mutation unlikely.

The T→C transition in codon 664 of the *RET* gene affects the protein kinase domain of the gene product in subdomain VIII, one of the major conserved subdomains of the protein kinases. In some of the subfamilies of protein tyrosine kinases, including the platelet-derived growth-factor receptor subfamily to which the *RET* protein (Ret) belongs, there is a methionine at the relevant peptide position in the kinase domain¹¹. In the remaining subfamilies, this position is occupied by a threonine in the large majority of cases. The protein serine/threonine kinase class shows a substantial diversity at this position, but no occurrence of threonine¹¹. Remarkably, the *RET* mutation in MEN 2B leads to the substitution of a threonine, mainly found in the other set of protein tyrosine kinases, for a methionine, normally found in the set to which Ret belongs. We therefore expect that the mutation causes some change in substrate specificity or perhaps in the mode of regulation rather than in catalytic function.

As protein tyrosine kinases do not occur in yeast and as a protein tyrosine kinase catalytic domain is part of many growth factor receptors, tyrosine specificity may have evolved in multi-cellular eukaryotes to play a role in cell-to-cell communication¹¹. For *RET*, this suggestion is corroborated by similarities between its extracellular receptor domain and cadherins, transmembrane proteins that mediate cell-cell adhesion¹². A critical role in mammalian embryogenesis, notably in migrating neural crest cells of the developing peripheral nervous and excretory system, is suggested by the results of *RET* expression studies and gene targeting experiments in mice¹³. From these observations we are now beginning to understand the various clinical symptoms of the syndromes in which Ret plays a role. □

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Point mutations affecting the tyrosine kinase domain of the *RET* proto-oncogene in Hirschsprung's disease

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HIRSCHSPRUNG'S disease is a genetic disorder of neural crest development affecting 1 in 5,000 births. It is characterized by the absence of intramural ganglion cells in the hindgut, which often results in partial to complete intestinal obstruction during the first years of life. An autosomal dominant gene causing this disease was recently mapped to chromosome 10q11.2 (refs 1, 2), using an interstitial deletion of this region isolated in a cell hybrid^{3,4}. It was subsequently localized to a 250-kilobase interval which contains the *RET* proto-oncogene⁵. Using flanking intronic sequences as primers⁶ to amplify 12 of the 20 exons of *RET* from genomic DNA of 27 Hirschsprung's disease patients, we have now identified four mutations (one frameshift and three missense) that totally disrupt or partially change the structure of the tyrosine kinase domain of the *RET* protein (Ret). Mutations in the extracellular cysteine-rich domain of Ret have been identified previously^{7,8} in patients with multiple endocrine neoplasia type 2A, and a targeted mutation in the tyrosine kinase domain of the same gene produces intestinal aganglionosis and kidney agenesis in homozygous transgenic mice⁹. Our results support the hypothesis that *RET*, in addition to its potential role in tumorigenesis, plays a critical role in the embryogenesis of the mammalian enteric nervous system.

The human *RET* proto-oncogene was identified and cloned through rearrangements that occurred *in vitro* during transfection experiments¹⁰ or in papillary thyroid carcinoma¹¹. *RET* was localized to chromosome 10q11.2 (ref. 12) in the same region to which Hirschsprung's disease (HSCR, or congenital megacolon) was subsequently mapped^{1,5}. Only recently has the genomic structure of *RET* been investigated^{6,13} and found to consist of 20 exons, the last one being subjected to alternative splicing. This splicing is well documented and leads to two isoforms of the protein, 1,072 and 1,114 amino acids, respectively¹⁴, which might have a tissue-specific distribution. The gene is at least 80 kilobase (kb) long (B.P. *et al.*, manuscript in preparation). Its extracellular calcium-binding domain is similar to that of the cadherins¹⁵, with a cysteine-rich region proximal to the transmembrane domain. Patients with multiple endocrine neoplasia type 2A (MEN 2A) have germ-line mutations in these cysteines which probably represent the first example of a dominant effect initiating an oncogenic mechanism in humans^{7,8}. Indirect evidence in favour of the dimerization of the Ret protein, obtained from the functional study of a Ret/PTC2 chimaeric protein^{16,17}, points to a dominant-negative oncogenic mechanism in MEN 2A. HSCR patients with complete deletion of a copy of the *RET* proto-oncogene show no sign of C-cell thyroid hyperplasia when subjected to a pentagastrin test⁵.

The molecular basis of the HSCR phenotype was therefore investigated by amplification using the polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) analysis of genomic DNA from 27 unrelated HSCR patients and 31 controls (see Fig. 1 legend for a description of the primers and amplification conditions used). All patients were

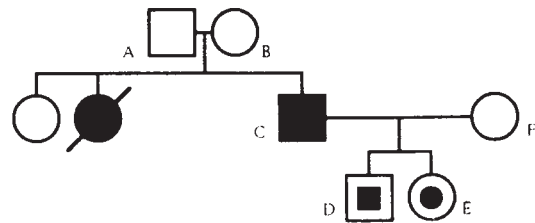


FIG. 1 Single-strand conformation polymorphism (SSCP) analysis of exon 17 in a pedigree with recurrence of long-form (■ or ●) and short-form (□ or ○) HSCR (variable expressivity). The mutation corresponding to the conformational variant revealed by SSCP is an Arg 972 → Gly substitution (Table 1). This mutation is found in three affected individuals as well as in an asymptomatic carrier (paternal grandmother), in keeping with the model of autosomal dominant transmission with incomplete penetrance, confirmed by linkage studies^{1,2}. The two heteroduplexes observed in all carriers of this mutation are indicated by an arrow. A–F, HSCR patient designations.

METHODS. Primers used for the four exons where mutations were identified. Exon 6: (forward) 5'-ATTGTTGTGCCCTACCTG3'; (reverse) 5'-CCCCAGACAGGCAATAGGTA3'. Exon 13 (forward) 5'-CTCTCTGTCTGAACTTGGGC3'; (reverse) 5'-TCACCTGCAGCAGGCCTTA3'. Exon 15: (forward) 5'-GACTCGTGCTATTTTCCTC3'; (reverse) 5'-TATCTTTCCTAGGC TTCCCT3'. Exon 17: (forward) 5'-TCACTGGTCCTTCACTCTCT3'; (reverse) 5'-GGGAGGGAATGCACACAGAT3'. PCR amplifications were carried out at 90 °C for 1 min, at 60 °C for 1 min and for 2 min at 72 °C for 30 cycles with 50 mM KCl, 10 mM Tris/HCl pH 8.3, 1.2 mM MgCl₂, 50 pmol primers, 200 μM of each deoxynucleotide triphosphate and 2.5 U *Taq* polymerase in a final volume of 50 μl. For SSCP analysis, all samples were run in 6% non-denaturing sequencing polyacrylamide gels under three different conditions: 10% glycerol at room temperature and 5% or 10% glycerol at 4 °C, modified from ref. 25. The size of the PCR products obtained can be deduced from our previous work⁶. DNA bands on the gel were visualized by silver staining.

classified according to criteria previously used by us¹, based essentially on the length of the aganglionic tract extending above (long-segment HSCR) or confined to below the splenic flexure (short-segment HSCR). Only 7 out of 27 patients had a long-form HSCR. Most patients (23/27) had sporadic HSCR. The SSCP screening performed for exons 3, 6, 8, 9, 10, 11, 13, 14, 15, 16, 17 and 18, using the flanking intronic sequences already identified⁶, indicated the presence of conformational variants in exons 3, 6, 11, 13, 15 and 17 of one or more patients and/or controls. Figure 1 shows a typical example of a conformational variant segregating in a HSCR family. Only four of these variants, found in three patients with sporadic occurrence and in one patient with familial recurrence (Fig. 1), were identified by sequencing as possible causative mutations. The remaining variants were silent mutations (either common polymorphisms or private variants), which will be reported elsewhere.

The three apparently sporadic mutations were found in patients with long-segment HSCR, whereas in the family with recurrence of HSCR, the mutation was associated with either the long-segment or short-segment form or with no symptoms at all (Fig. 1). Five of the six parents of the three sporadic cases

TABLE 1 Mutations associated with HSCR

	Mutation	Deletion and codon substitution	Occurrence
Two independent cytogenetically visible deletions	del10q11.2-21.2	Complete deletion of <i>RET</i> ^{3,5}	Sporadic
Deletion not visible cytogenetically	Microdeletion in 10q11.2	Complete deletion of <i>RET</i> ⁵	Familial
Exon 6	1,120 del G	Frameshift after the first 373 amino acids	Familial
Exon 13	T → C (2,293)	Ser 765 → Pro	Sporadic
Exon 15	G → A (2,690)	Arg 897 → Gln	Sporadic
Exon 17	A → G (2,914)	Arg 972 → Gly	Familial

Mutated nucleotides are defined on the basis of the complementary DNA sequence, starting from ATG. Mutations in exons 6 and 15 were identified by direct sequencing carried out on purified amplification products (containing both the normal and the mutated alleles) using the Sanger dideoxy-mediated chain-termination method²⁴ with Sequenase version 2.0 (US Biochemicals, Cleveland). Mutations in exons 13 and 17 were identified after cloning the PCR products using the pCRTM II vector (TA Cloning Kit; Invitrogen).

were tested for the mutation identified in their children and one of them was found to be a silent carrier of the frameshift mutation in exon 6 (Table 1) which causes early termination of translation at nucleotide 1,355, where a new stop codon arises. The three missense mutations (Table 1) might result in inactivation of the Ret because of the substitution of highly conserved amino acids like the arginine residue of codon 897 mutated to glycine, which is conserved in at least 90% of the protein tyrosine kinases¹⁸. In contrast, the remaining two missense mutations do not affect highly conserved amino acids¹⁸.

These three missense mutations occur in the tyrosine kinase domain of Ret and might have a loss-of-function effect equivalent to those of the frameshift mutation and the deletions reported previously⁵. The problem then arises of explaining the dominant effect caused by the loss of one copy of the *RET* proto-oncogene.

Mutations in the murine *c-kit/white* spotting locus which, like *RET*, encodes a tyrosine kinase receptor, lead to different phenotypes that can be explained on the basis of either dominant-negative or loss-of-function effects^{19,20}. Functional or structural loss of one copy of *RET* (dosage effect) might play a similar critical role in development and offers a unifying molecular explanation for the different types of mutations observed in HSCR patients (Table 1). In a similar way, the human equivalent of the white spotting mutant, that is, the autosomal dominant piebald trait, can be caused either by complete deletion²¹ of the *KIT* proto-oncogene or by a point mutation in its tyrosine kinase domain²². Finally, the targeted mutation in the tyrosine kinase domain of murine *ret* behaves like a recessive gene, causing total intestinal aganglionosis in homozygous mice but not in heterozygotes⁹. This discrepancy between apparently similar mutations in humans and mice might be due to the effect of one or more modifier gene(s) whose action is more marked in humans than in inbred mouse strains.

The efficiency in identifying *RET* mutations in the present work may be quantified in the following way. We analysed 12 exons, or 55% of the entire coding sequence (1,857 out of 3,374 base pairs (bp)). If the efficiency of detecting mutations by SSCP is of the order of 50%, one would expect to find mutations in 28% of patients (that is, eight instead of the four observed). This discrepancy might be accounted for by other genes producing the same phenotype (genetic heterogeneity). However, this is not supported by linkage studies which indicate that *RET* does not recombine with HSCR in any of the pedigrees studied so far (our unpublished results). In conclusion, *RET* seems to be the major gene causing HSCR. The relatively low efficiency in

detecting mutations might be due to possible, so far unidentified, clusters as observed in MEN 2A^{7,8} and MEN 2B²³, and/or to technical reasons inherent to SSCP. □

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Mutations of the *RET* proto-oncogene in Hirschsprung's disease

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HIRSCHSPRUNG'S disease (HSCR)¹ is a common condition (1 in 5,000 live births) resulting in intestinal obstruction in neonates² and megacolon in infants and adults³. This disease has been ascribed to the absence of autonomic ganglion cells, which are derived from the neural crest, in the terminal hindgut⁴. Segregation analyses have suggested incompletely penetrant dominant inheritance in familial HSCR⁵. Recently, a gene for HSCR has been mapped to chromosome 10q11.2 (refs 6, 7). No recombination was observed between the disease locus and the locus for the *RET* proto-oncogene⁸, a protein tyrosine kinase gene expressed in the cells derived from the neural crest^{9,10}. Here we report nonsense and

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missense mutations in the extracellular domain of *RET* protein (exons 2, 3, 5 and 6) in six unrelated probands and show that the mutant genotypes segregate with the disease in HSCR families. Mutations of *RET* have been previously reported in multiple endocrine neoplasia type 2A (MEN 2A)^{11,12}. Thus, germ-line mutations of the *RET* gene may contribute either to developmental anomalies in HSCR or to inherited predisposition to cancer in MEN 2A.

Hirschsprung's disease (aganglionic megacolon, HSCR)¹ is a congenital malformation thought to result from the premature arrest of the cranio-caudal migration of neural crest cells in the wall of the developing gut (weeks 5–12 of gestation)¹³. Several lines of evidence suggest the involvement of genetic factors in this disease¹⁴, including: an elevated risk to siblings (4–19%)¹⁵; a dominant pattern of inheritance in several HSCR pedigrees¹⁶; the association of HSCR with malformative syndromes¹⁷ and with chromosomal anomalies^{18,20}; and mendelian inheritance of colonic aganglionosis in rodents²¹.

Recently, we have mapped a gene for familial HSCR to chromosome 10q11.2 in the interval defined by loci *D10S208* and *D10S196* (ref. 6), and have shown that the disease gene is tightly linked to the *RET* proto-oncogene locus (maximum pairwise loci score $Z_{\max} = 7.54$, with no recombination in 19 HSCR families; ref. 8, and P.E. *et al.*, manuscript in preparation). On the other hand, mutations of *RET* have recently been identified as the underlying cause of an inherited cancer syndrome, multiple endocrine neoplasia type 2A (MEN 2A)^{11,12}, which is characterized by tumours of cells derived from the neural crest. Together, these data suggested *RET* as a candidate gene for HSCR.

Preliminary analyses indicated that no major deletion or rearrangement of the *RET* locus had occurred in our series (not shown). Our strategy, therefore, was to analyse the coding sequence of *RET* for point mutations or minute changes using a combination of single-strand conformation polymorphism (SSCP)²² and direct sequencing analyses of nine exons encoding the extracellular, transmembrane and tyrosine kinase domains of the protein²³ in patients with familial HSCR. Abnormal patterns of migration, suggesting sequence variation in the extracellular domain (exons 2, 3, 5, 6), were observed in probands from 4 out of 19 families linked to chromosome 10q11.2, and in 2 of 15 additional families insufficient for linkage (families 20 and 21, Fig. 1). SSCP variants were not detected in exon 4, in exons 10 and 11 which span the transmembrane domain and encompass the cysteine-rich region mutated in MEN 2A (Fig. 1), nor in exons 12 and 14 which include the consensus ATP-binding sites. Direct sequence analysis in the six probands showed heterozygosity for missense or nonsense mutations, predicted to result in amino-acid substitution or protein termination in the extracellular domain of *RET* protein (Ret, Table 1). The base substitutions which frequently involved CpG doublets (3/6)²⁴

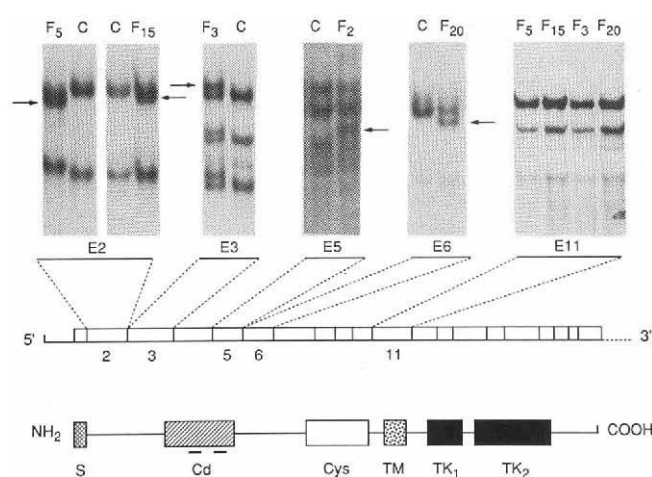


FIG. 1 Single-strand conformation polymorphism (SSCP) analysis of *RET* exons 2, 3, 5, 6 and 11 in HSCR probands. The arrows indicate the abnormal bands in probands. F, Patient from indicated family; C, control. The exons (E) showing abnormal SSCP patterns (exons 2, 3, 5, 6) and the corresponding functional domains of the *RET* protein are indicated. S, Signal sequence; Cd, cadherin-like domain; TM, transmembrane domain; Cys, cysteine-rich domain; TK, tyrosine kinase domains 1 and 2. Calcium-mediated binding sites are underlined.

METHODS. Based on the nucleotide sequence of the exon-intron boundaries of the gene²³, we devised oligonucleotide primers for the polymerase chain reaction (PCR) amplification of nine large exons spanning 56% of the *RET* coding sequence. Exon 2: A-5'-AGTGGCATGGG-CCTCTAC-3', B-5'-TGCGGACACTGAGCTTCTC-3' (266 base pairs). Exon 3: A-5'-ACCGCGGCTTCCCTGCT-3', B-5'-CCTCCAGGAGCCTGTAGGC-3' (288 bp). Exon 5: A-5'-GACACCGTGGTGCCACGC-3', B-5'-TATAGTCATGATCGGTGCG-3' (196 bp). Exon 6: A-5'-GCTGGTCTCAACCGGAAC-3', B-5'-CTGGGCAATCGCGAGCC-3' (200 bp). Exon 11: A-5'-GCATACGCAGCCTGTACCC-3', B-5'-AAGCTTGAAGGCATCCACGG-3' (320 bp). Genomic DNA samples were amplified in the presence of dCTP P³²* (NEN) using exonic primers, except for exon 11. The PCR products were heated for 10 min at 95 °C, loaded onto a Hydrolink MDE gel (Bioprobe) and electrophoresed for 18 h at 4 W. The gels were dried and autoradiographed.

were absent in 45 normal controls ($P < 0.001$).

Additional evidence that mutation of the *RET* gene is significant in HSCR was provided by the co-segregation of the observed base substitution with the disease in each of five families. Figure 2A shows that the Ser 32 → Leu (S32L) mutation (exon 2) in family 5 abolished a *TaqI* restriction site in all affected individuals and obligate carriers, but not in other family members or controls. Similarly, the mutant DNA sequence for each of families 2, 3, 15 and 20 also co-segregated with the disease (Fig. 2B).

Although all 19 HSCR families are linked on chromosome 10q11.2 in our series, *RET* mutations were detected in only four of these and in two additional smaller families. The role of *RET* mutations in the remaining kindreds and in sporadic HSCR remains to be determined.

Several lines of evidence suggest that the mechanism of *RET* involvement in the HSCR phenotype is the result of gene inactivation or abrogation of the functional *RET* product. First, deletions of chromosome 10q11.2 associated with HSCR have been reported. In each case, the deletion spans the *RET* locus^{20,25}. Second, we have identified two patients with *RET* nonsense mutations, resulting in a premature stop codon, predicted to produce a very truncated and, presumably, non-functional *RET* protein. Finally, the *RET* HSCR missense mutations involved amino-acids conserved in mouse²⁶ and were scattered in the putative ligand-binding region of Ret, consistent with the diversity of events predicted to be associated with gene inactivation. Interestingly, one of the six mutations identified in HSCR patients was in exon 5, which encodes a Ret domain with homology to the cadherin family of cell-adhesion molecules^{26,27}. Disruption of

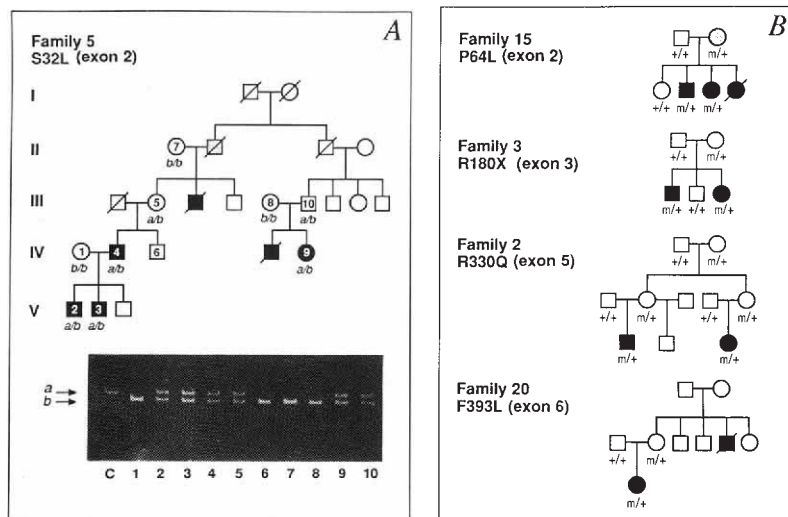
TABLE 1 *RET* mutations in HSCR families

Exon	Family	Nucleotide change (<i>RET</i> codon)	Effect on protein sequence	Mutation
2	5	TCG → TT*G (32)	Ser → Leu	S32L
2	15	CCC → CT*C (64)	Pro → Leu	P64L
3	21	GAG → T*AG (136)	Glu → Stop	E136X
3	3	CGA → T*GA (180)	Arg → Stop	R180X
5	2	CGG → CA*G (330)	Arg → Gln	R330Q
6	20	TTC → TTA* (393)	Phe → Leu	F393L

Codon and amino-acid positions are numbered as described for the full-length coding sequence^{23,30}.

* Nucleotide change.

FIG. 2 Segregation of the mutant alleles in HSCR families. A, Segregation of the S32L mutation in family 5. The S32L mutation abolished a *TaqI* restriction site in exon 2. All affected individuals and obligate carriers show a doublet corresponding to the mutant allele *a* (266 bp) and the normal allele *b* (242 bp). Other family members display a single 241-bp fragment (*b/b*). C, undigested amplification product of a control DNA. Genomic DNA was PCR-amplified with primers of exon 2, digested with restriction enzyme *TaqI* and electrophoresed on a PCR Purity Plus gel (Bioprobe). B, Segregation of the P64L, R180X, R330Q and F393L mutations in HSCR families. The segregation of mutant genotypes was studied using either the SSCP technique or direct sequencing. m, Mutant allele; +, normal allele. In each family the mutant allele segregated with the disease. Phenotype definition: histopathological criteria for HSCR were: increased acetylcholinesterase histochemical staining in nerve fibres on suction biopsies of the rectal submucosa and absence of submucosal and myenteric neuronal ganglia on histological evaluation of the aganglionic tract. Two individuals with severe constipation starting in childhood were considered as affected (shaded symbols). Atypical forms associated with intestinal neuronal dysplasia, hearing loss, dysmorphic features, pigmentary disorders, malformations or chromosomal anomalies were excluded.



cell-to-cell interactions modulated by this cadherin-like motif is an attractive model for the failure of neural crest cell migration observed in HSCR^{13,28}. In contrast, point mutations of the *Ret* extracellular domain responsible for MEN 2A and familial medullary thyroid carcinoma^{11,12} are clustered in only five cysteine codons, consistent with alterations of the protein leading to activation of the *RET* tyrosine kinase²⁹.

Our data suggest that germ-line mutations of the *RET* proto-oncogene can lead to very different phenotypes affecting cells derived from the neural crest. The developmental abnormalities observed in HSCR seem to result from inactivation or abrogation of the *RET* product, whereas predisposition to neoplasia, as seen in MEN 2A and familial medullary thyroid carcinoma, may result from activating mutations. □

Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor *Ret*

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RECEPTOR tyrosine kinases (RTKs) are cell-surface molecules that transduce signals for cell growth and differentiation¹. The RTK encoded by the *c-ret* proto-oncogene²⁻⁵ is rearranged and constitutively activated in a large proportion of thyroid papillary carcinomas⁶, and germ-line point mutations in *c-ret* seem to be responsible for the dominantly inherited cancer syndromes multiple endocrine neoplasia (MEN) types 2A^{7,8} and B⁹. The gene is expressed in the developing central and peripheral nervous systems (sensory, autonomic and enteric ganglia) and the excretory system (Wolffian duct and ureteric bud epithelium) of mice, indicating that it may play a role in normal development⁵. Here we show that mice homozygous for a targeted mutation in *c-ret* develop to term, but die soon after birth, showing renal agenesis or severe dysgenesis, and lacking enteric neurons throughout the digestive tract. *Ret* is thus an essential component of a signalling pathway required for renal organogenesis and enteric neurogenesis.

A targeting construct (Fig. 1A) was designed to insert a *neo^R* gene into the *c-ret* gene, simultaneously deleting 0.8 kilobases (kb) of DNA, including the codon for an invariant lysine required for kinase activity¹⁰. The construct was introduced into embryonic stem (ES) cells, and two clones containing the predicted mutant allele (called *ret-k⁻* for *ret*-kinase minus) were

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identified (Fig. 1B). When injected into mouse blastocysts, one clone (clone 199) produced chimaeric males that transmitted the mutation through the germ line. Offspring heterozygous for *ret-k*⁻ seemed to be normal, whereas *ret-k*⁻ homozygotes (Fig. 1C, D) were born in the expected proportion (79/304) and appeared normal at birth, but all died within 16–24 h of birth. Dissection of these mice revealed that the kidneys were either absent or rudimentary (Fig. 2). Histological analysis of mutant kidney rudiments (Fig. 3b–d) showed severe dysplasia, with reduced numbers of recognizable nephric elements (including proximal and distal tubules, glomeruli and vessels), which were randomly distributed with no recognizable medulla, cortex or nephrogenic zone (compare with Fig. 3a), reduced branchings of the ureter without formation of mature collecting ducts (Fig. 3b), and large regions of undifferentiated mesenchyme. Some homozygotes contained blind-ending ureters with no renal tissue, and others displayed a complete absence of ureter and kidney (renal agenesis), either unilateral or bilateral (Fig. 2).

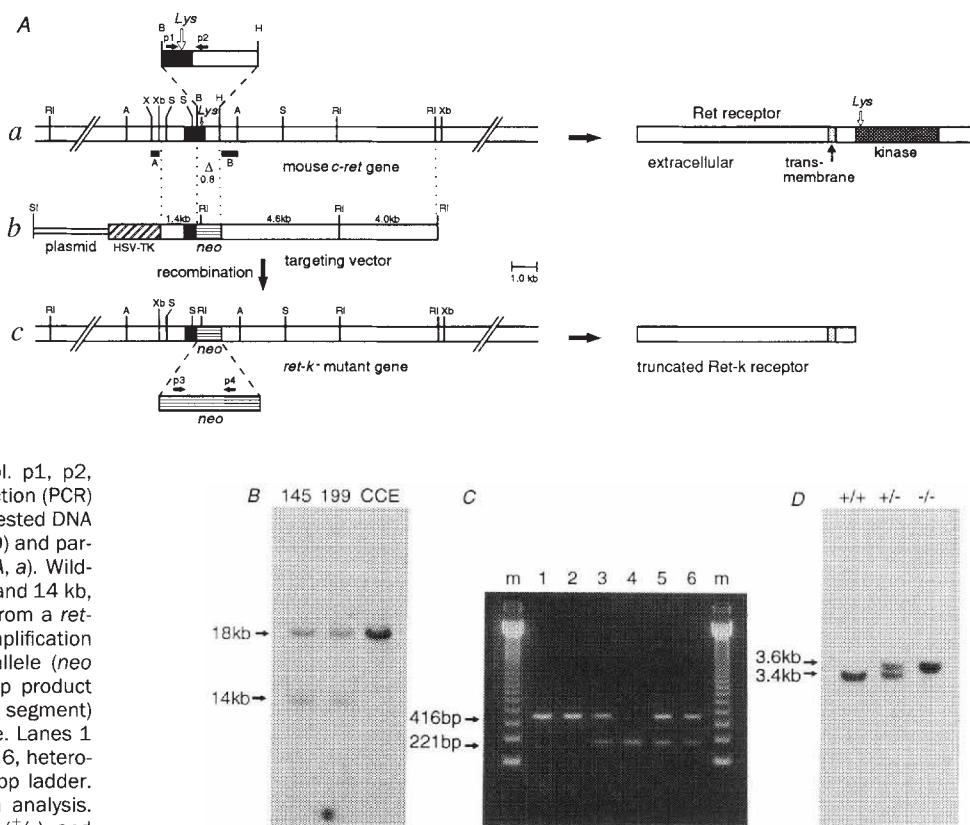
Normal kidney development involves reciprocal inductive interactions between the ureteric bud, an outgrowth of the Wolffian duct, and the adjacent metanephric mesenchyme¹¹. The mesenchymal cells induce the ureteric bud to grow and branch repeatedly, forming the renal collecting system^{12,14}, and the ureteric bud induces the mesenchyme to condense into epithelial structures which differentiate into the glomeruli and proximal and distal tubules^{11,13}. Based on our findings, we propose that the Ret receptor normally transduces a mesenchyme-derived signal that stimulates the formation, growth and branching of the ureteric bud. The hypothesis is consistent with the sites of expres-

sion of the *c-ret* gene (the ureteric bud and its derivatives, but not the mesenchymal compartment)⁵ and with the *ret-k*⁻ mutant phenotype. The histology of the rudimentary kidneys observed in some *ret-k*⁻ mice seemed to be consistent with failure of the ureteric bud to grow and branch appropriately. Furthermore, the most frequent excretory defect, renal agenesis, seems to result from failure of the ureteric bud to form in the E11.5 embryo (our unpublished data). Recent observations on mice with a mutation in the Wilms' tumour gene (*WT1*) implied that a signal from the metanephric mesenchyme is required to induce the initial formation of the ureteric bud¹⁵. Our results, combined with the expression of *c-ret* in the posterior Wolffian duct before the outgrowth of the ureteric bud, support this model and suggest that *c-ret* encodes the receptor for this inductive signal.

The second abnormality in all mutant homozygous pups was a failure of milk to progress from the stomach into the intestine, indicating a defect in gastrointestinal peristalsis. As the normal *c-ret* gene is expressed in the developing enteric ganglia⁵, this suggested that the mutation affected the development of the enteric nervous system (ENS). Histological analysis revealed that the neurons of the myenteric plexus (arrows in Fig. 3e, g) were absent from the small and large intestine (Fig. 3f, h), as well as from the oesophagus and stomach (data not shown for mutant animals). These observations were confirmed by immunofluorescence studies using antibodies against neuron-specific enolase and peripherin, two proteins expressed in enteric neurons^{16,17}. Whereas many neuron-specific enolase- and/or peripherin-positive cells were observed in the myenteric and submucosal plexuses of the small and large intestine from hetero-

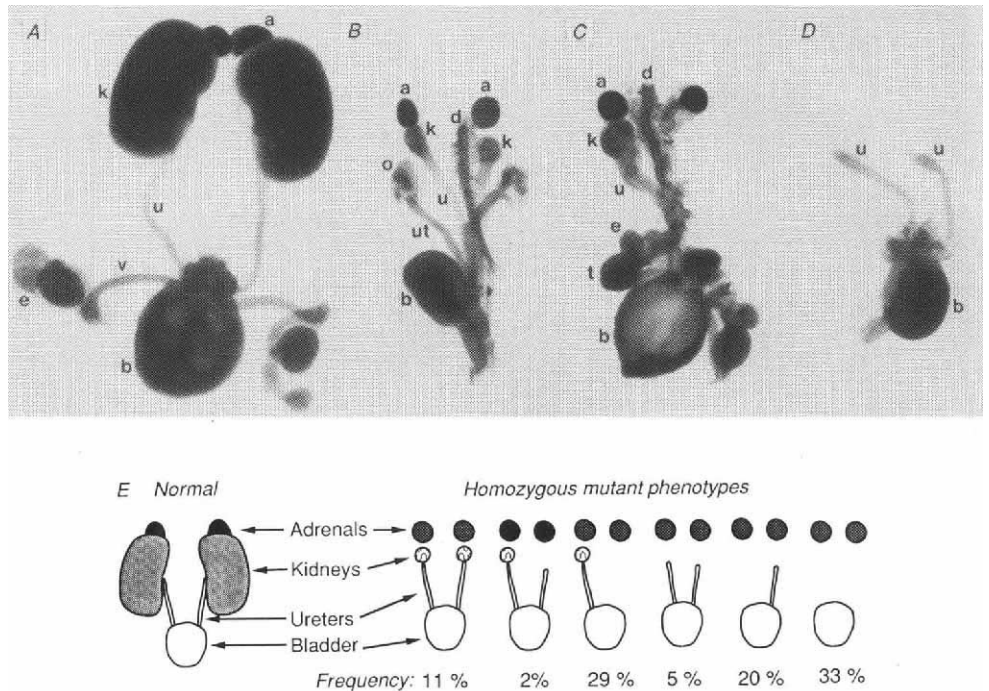
FIG. 1 Targeting the murine *c-ret* locus.

A, Targeting strategy. **a**, A segment of the normal *c-ret* gene (strain 129), including an exon (black box) containing the codon for the essential Lys 748 residue⁴ in the kinase domain. **b**, Targeting vector, including two segments of *c-ret*, the pMC1neoPA (*neo*) and HSV-TK genes³¹. **c**, Mutant *ret-k*⁻ locus, in which *neo* has replaced 0.8 kb of the *c-ret* gene. Transcripts terminating at the *neo* polyadenylation site should encode a truncated receptor lacking the kinase domain, and any transcripts proceeding through *neo* would encode an inactive kinase lacking Lys 748. Restriction enzyme sites: A, *Apal*; B, *BamHI*; H, *HincII*; Rl, *EcoRI*; S, *SacI*; Sl, *Sall*; Xb, *XbaI*; X, *XhoI*. p1, p2, p3 and p4 indicate polymerase chain reaction (PCR) primers. **B**, Southern analysis of *EcoRI*-digested DNA from targeted ES cell clones (145 and 199) and parental cells (CCE), hybridized to probe A (A, a). Wild-type and mutant alleles yield bands of 18 and 14 kb, respectively. **C**, PCR analysis of progeny from a *ret-k*⁻/+ intercross. The 416-base-pair (bp) amplification product is generated from the mutant allele (*neo* primers p3 and p4), whereas the 221-bp product (primers p1 and p2 within 0.8-kb deleted segment) is generated only from the wild-type allele. Lanes 1 and 2, homozygous *ret-k*⁻; lanes 3, 5 and 6, heterozygous *ret-k*⁻; lane 4, wild type. m, 123-bp ladder. **D**, Verification of genotypes by Southern analysis. DNA from wild-type (+/+), heterozygous (+/-) and homozygous mutant (-/-) mice were digested with *SacI* and hybridized with probe B (A, a). The wild-type allele generates a 3.4-kb band and the mutant allele a 3.6-kb band. Disruption of the *c-ret* gene was confirmed at the messenger RNA level by northern analysis of RNA from brains of homozygous *ret-k*⁻ pups; the normal *c-ret* transcripts⁵ were absent, and were replaced by several transcripts of very low abundance whose sizes were consistent with predicted products of the mutant locus (data not shown).



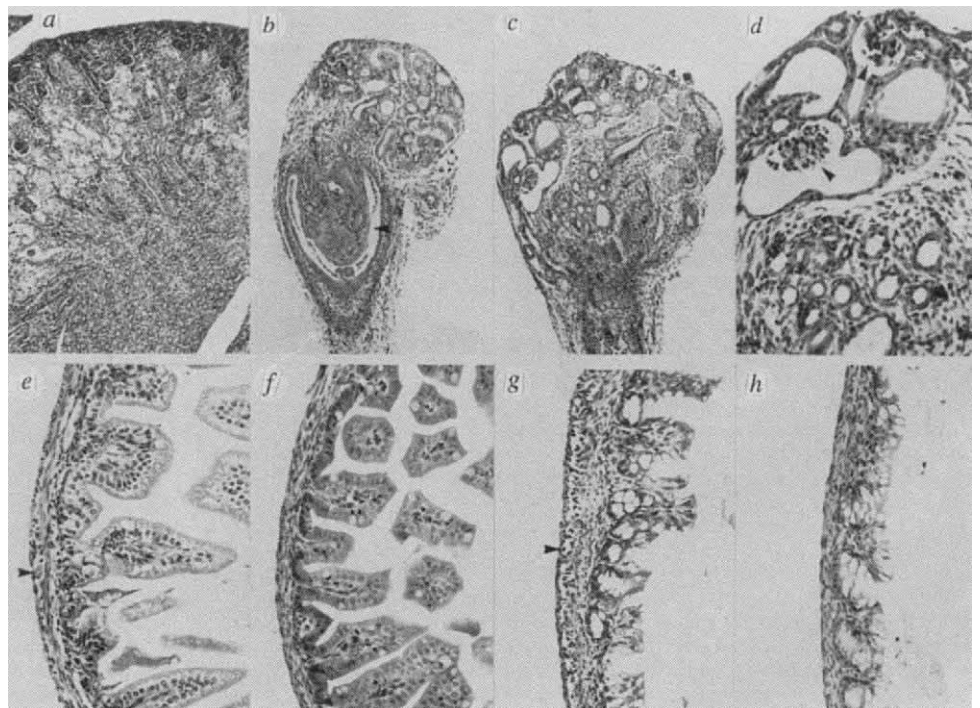
METHODS. The targeting vector was linearized and introduced into CCE ES cells³² by electroporation. The cells were selected with G418 and gancyclovir³¹ and 200 surviving clones were analysed. Chimaeric male mice were produced by blastocyst injection³³ and mated to strain MF1 females, and heterozygous mutant progeny were intercrossed to produce subsequent generations. Details of the vector construction and PCR conditions are available on request.

FIG. 2 Dissected urogenital systems from wild-type and mutant newborn mice. A, Urogenital system dissected from a wild-type newborn male mouse. B–D, Urogenital system dissected from three *ret-k⁻* homozygous newborn mice, showing some of the phenotypes observed, all at the same magnification as A. B, Urogenital system dissected from a mutant female newborn mouse which had two kidney rudiments connected by ureters to the bladder. C, Urogenital system dissected from a mutant male newborn mouse, displaying a kidney rudiment and ureter on the left, but neither a ureter nor a kidney on the right. D, A specimen with two blind-ending ureters emerging from the bladder. No renal tissue could be observed at the tips of the ureters even after serial sectioning and histological analysis of the entire urogenital tissue block (not shown). a, Adrenal; k, kidney; u, ureter; v, vas deferens; e, epididymis; t, testes; b, bladder; d, dorsal aorta; o, ovary; ut, uterine horn. E, Frequency of kidney rudiments and blind-ending ureters among homozygous *ret-k⁻* newborn mice. On the left is a diagram of the excretory system of the wild-type newborn mice; on the right are diagrams and frequencies of the phenotypes observed in the mutant newborn progeny of outbred *ret-k⁺* intercrosses ($n = 55$). The reproductive organs seemed to be normal in all males (A, C) and most females (B). But about 25% of homozygous mutant females displayed uterine abnormalities, including absence of the caudal end



of one uterine horn, or fusion of one uterine horn with the ipsilateral ureter (not shown). Although this finding was unexpected, as expression of *c-ret* was not detected in the müllerian duct⁵, abnormalities of müllerian duct derivatives are among the most common anomalies associated with renal agenesis in humans³⁴.

FIG. 3 Histological analysis of wild type compared with *ret-k⁻* mutant kidneys and gastrointestinal tract. a, Normal kidney, showing the normal organization into nephrogenic zone (top), cortex and medulla (lower right). Magnification, $\times 38$. b, c, Two different sections through a mutant kidney rudiment, photographed at the same magnification as a. Note the disorganized parenchyma and the dilated tubules and Bowman's capsules. The arrowhead in b indicates the ureter, which has entered the kidney and branched to form the pelvico-calyceal system. Magnification, $\times 38$. d, Higher magnification of a region from c. The arrowheads point to two glomerular tufts. Also visible at this magnification is the abundant, primitive mesenchyme which is observed in mutant, but not wild-type newborn kidneys. Magnification, $\times 95$. e–h, Sections through the gastrointestinal tracts of wild-type (e, g) and homozygous mutant (f, h) newborn mice. e, f, Small intestine; g, h, large intestine. The arrowheads in e and g point to neurons of the myenteric plexus, which are identified by their large size, clear cytoplasm and prominent nuclei. In the homozygous mutant mice, the ganglion cells are absent and the thickness of the longitudinal and circular muscle layers is reduced. Magnification, $\times 95$.



METHODS. Embryos were fixed in 10% formalin, dehydrated and embedded in paraffin. Sections ($4\mu\text{m}$) were cut and stained with haematoxylin and eosin.

zygous (*ret-k*⁻/+) animals, no staining was seen in the corresponding sites in homozygous mutant animals (Fig. 4).

The majority of the neurons and glia of the ENS are derived from the vagal neural crest, which migrates from the postotic hindbrain along predetermined pathways to populate the entire length of the bowel^{18,19}. Despite the unique properties of the ENS, neural crest cells that do not normally generate enteric neurons or glia have the potential to form a normal and functional ENS, containing bowel-appropriate neuronal and glial phenotypes when grafted to ectopic sites in avian embryos^{20,21}. These observations established that the microenvironment of the embryonic bowel is capable of directing uncommitted neural crest precursors towards ENS-specific phenotypes²². We demonstrate here that *c-ret* plays a critical role in the establishment of the enteric neuronal lineage. Although the mechanism of action of *c-ret* remains unknown, we propose that it encodes the receptor for a signalling molecule required for the migration or

differentiation of the vagal neural crest and its derivatives. The identity of this molecule(s) and its relationship to the Ret-mediated signal involved in renal organogenesis are unknown.

c-ret is normally expressed in other parts of the developing peripheral nervous system (including cranial, dorsal root and sympathetic ganglia) as well as in motor neurons of the spinal cord and brain⁵. Although preliminary histological analysis revealed no other anatomical abnormalities of the peripheral or central nervous systems, further experiments will be necessary to determine whether other sites are affected by the *ret-k*⁻ mutation.

Our findings suggest that mutations in the human *RET* gene may be responsible for hereditary defects of the excretory and enteric nervous systems. Renal agenesis or dysgenesis occurs at a frequency of 1 in 3,000–6,000 births, including sporadic and familial cases^{23,24}. Although no genetic loci associated with these defects have yet been identified, mutations or deletions of the *RET* locus could be responsible for a fraction of these cases. The most common congenital defect (1 in 5,000 births) affecting the development of the ENS in humans is Hirschsprung's disease (HSCR; aganglionic megacolon). HSCR is characterized by the absence of enteric ganglia from the hindgut, and although the histopathological abnormalities are usually restricted to the sigmoid colon, some cases also involve the proximal colon and even the distal small intestine²⁵. Recent physical mapping and genetic linkage studies have independently raised the possibility that mutations in the *RET* locus are responsible for aganglionic megacolon in humans, having co-localized HSCR and *RET* to an interval of ~250 kb on chromosome 10 (refs 26–28). This suggestion is further supported by the identification of specific mutations in the *RET* gene co-segregating with the disease phenotype in HSCR families^{29,30}. These genetic studies are consistent with our analysis of the effect of the *ret-k*⁻ mutation on the ENS, and indicate that the *ret-k*⁻ mutant mice represent a useful animal model for study of the developmental defects underlying HSCR. Overall, the mutations of the *c-ret* proto-oncogene described so far provide a unique opportunity to study the varying *in vivo* effects of multiple alleles of a RTK gene in tumour formation and normal mammalian development. □

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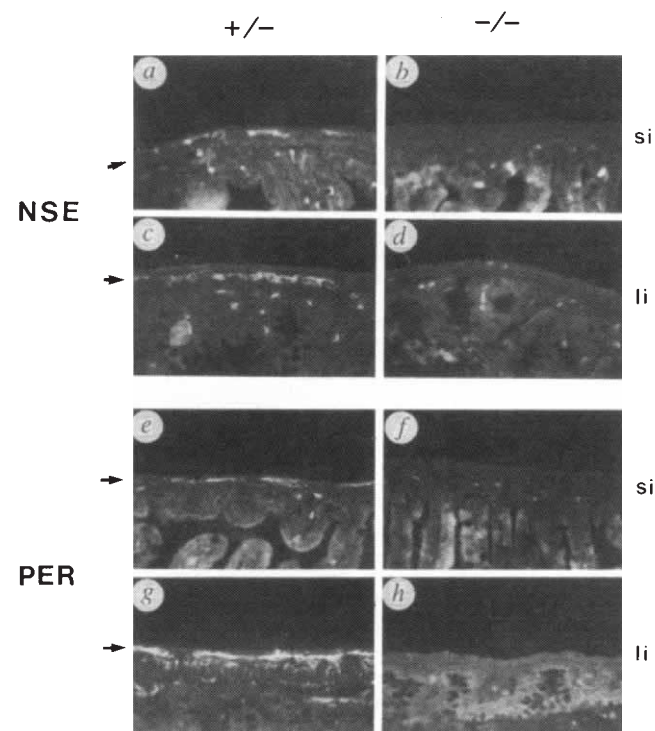


FIG. 4 Absence of neurons from the *ret-k*⁻ mutant gut wall. Immunofluorescence micrographs showing sections of the small intestine (si) and large intestine (li) of heterozygous *ret-k*⁻/+ and homozygous *ret-k*⁻/*ret-k*⁻ newborn mice stained with antibodies against neuron-specific enolase (NSE; a–d) and peripherin (PER; e–h). Neurons of the myenteric plexus are present in the gut wall of the small and large intestine of heterozygous mice (arrows), but are absent from the gut wall of homozygous mutant animals. Peripherin-positive neurons of the submucosal plexus are also visible in the large intestine of heterozygotes (g), but are absent in homozygotes (h). Positive-staining cells in homozygous mutant intestine are not in the position of the myenteric or submucosal plexuses, and may be neuroendocrine cells or axons of extrinsic neurons innervating the gut.

METHODS. Small and large intestines from newborn mice were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C for 4–6 h. Frozen 10-μm sections were mounted onto gelatin-coated slides and washed with PBS. The neurons in the enteric ganglia were identified with rabbit antibodies against NSE (Polysciences) and peripherin (provided by R. Liem). Sections were incubated in primary antibodies overnight at 4 °C. After washing with PBS, sections were incubated with fluorescein isothiocyanate-conjugated sheep anti-rabbit immunoglobulin (a gift from R. Morris) for 2 h at 22 °C, and finally washed twice with PBS. Sections were examined on a Zeiss Axiophot microscope equipped with epifluorescence optics. With each antibody, at least five homozygous and five heterozygous newborn mice were analysed, and at least five sections of small intestine and five of large intestine were examined.

Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene

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Host genotype specificity in interactions between biotrophic pathogens and plants in most cases complies with the gene-for-gene model¹; success or failure of infection is determined by absence or presence of complementary genes, avirulence and resistance genes, in the pathogen and host plant, respectively. Resistance, expressed by the induction of a hypersensitive response in the host, is envisaged to be based on recognition of the pathogen, mediated through direct interaction between products of pathogen avirulence genes (the so-called race-specific elicitors) and receptors in the host plant, the putative products of resistance genes¹. The interaction between the biotrophic fungus *Cladosporium fulvum* and its only host, tomato (*Lycopersicon esculentum*), is a well-established model system for studying fungus-plant gene-for-gene relationships¹. Here we report the isolation of race-specific elicitor AVR4 of *C. fulvum* and the cloning of its encoding avirulence gene. We present evidence that, in nature, a single base-pair change in this avirulence gene leads to virulence of races previously avirulent on tomato genotypes carrying the complementary *Cf4* resistance gene.

Race-specific protein elicitor AVR4, which specifically induces a hypersensitive response in tomato genotypes carrying resistance gene *Cf4*, is present in apoplastic fluid² isolated from a compatible interaction between race 5 of *C. fulvum* and tomato genotype *Cf5* (Table 1). Fractionation of the proteins present in apoplastic fluid revealed that the specific hypersensitive-response-inducing activity resided in a protein of relative molecular mass (M_r) 12,000 (Fig. 1a). The N-terminal amino-acid sequence of the protein was determined and a degenerated oligonucleotide probe was designed that allowed the isolation of three full-length AVR4-encoding complementary DNA clones (Fig. 1b). The elicitor is encoded as a pre-pro-protein of total length 135 amino acids, from which a putative N-terminal signal peptide is cleaved upon extracellular targeting³ (Fig. 1b). The resulting protein of 117 amino acids is processed into the mature elicitor by plant and/or fungal proteases, a phenomenon also observed for the AVR9 elicitor of *C. fulvum*⁴. The mature AVR4 elicitor contains eight cysteine residues and shows no significant homology to protein sequences present in the SwissProt database. Northern blot analysis revealed that expression of the *avr4* gene is specifically induced *in planta*; an *avr4* transcript was detected from 6 days after inoculation in the compatible *Cf5*/race 5 interaction, and a similar transcript was also present in the compatible *Cf4*/race 4 interaction (race 4 of *C. fulvum* does not produce the AVR4 elicitor; Table 1, and results not shown).

Southern analysis of genomic DNA isolated from races of *C. fulvum* avirulent (races 2, 5 and 2.5.9) or virulent (races 4, 2.4, 2.4.5 and 2.4.9.11) on tomato genotype *Cf4* revealed that all races contain a homologous, single-copy gene that displays no restriction-fragment length polymorphism (results not shown). This prompted us to investigate whether the *avr4* gene is indeed responsible for avirulence of *C. fulvum* on tomato genotype *Cf4*. Thus, a genomic *avr4* clone was isolated and used to transform race 4 of *C. fulvum*. Southern blot analysis of two hygromycin-resistant transformants (transformants A and B, Fig. 2a) showed

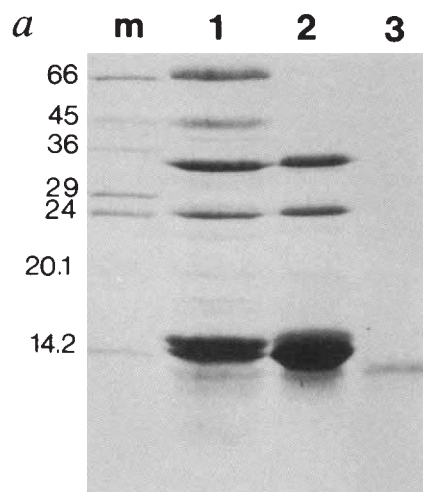


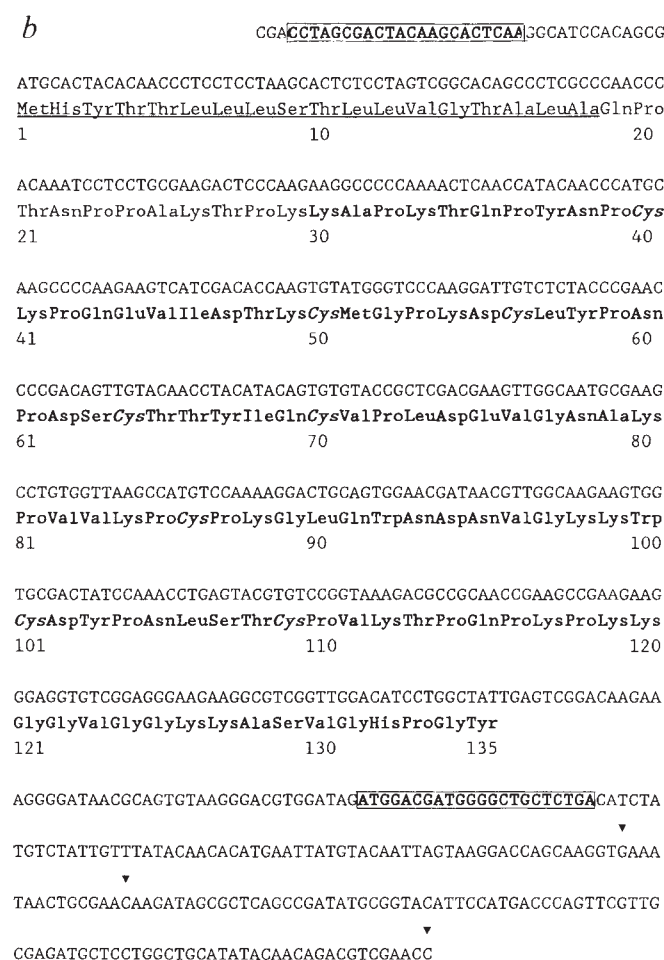
FIG. 1 Purification of the race-specific AVR4 protein elicitor and structure of *avr4* cDNA. a, SDS-polyacrylamide gel electrophoresis of protein fractions obtained on purification of race-specific protein elicitor AVR4. Lane 1, apoplastic fluid from tomato genotype *Cf5* infected by race 5 of *C. fulvum* (50 μ g protein loaded); lane 2, elicitor-active protein fraction obtained on gel filtration (50 μ g protein loaded); lane 3, purified AVR4 elicitor (3 μ g protein loaded). The lane marked 'm' contains M, markers of 66,000–14,200. b, DNA and protein sequence of the AVR4 elicitor as determined from *avr4* cDNA. Polyadenylation sites observed in three individual cDNA clones are indicated by closed triangles above the DNA sequence. The primers used for PCR analysis of the various races of *C. fulvum* are shown boxed in the DNA sequence. The putative signal peptide of the encoded protein is underlined and the sequence of the mature AVR4 elicitor protein is indicated in bold type.

METHODS. Proteins present in apoplastic fluid, obtained from leaves of tomato genotype *Cf5* infected by race 5 of *C. fulvum*², were concentrated by precipitation in 60% acetone and subjected to gel filtration¹². Fractions were assayed for hypersensitive-response-inducing activity by injection into leaflets of tomato genotype *Cf4*, which were examined for chlorosis and/or necrosis the next day. Biologically active fractions were loaded on a ProRPC HR 5/10 reversed-phase column (Pharmacia) and eluted by a gradient from 10% to 30% acetonitrile in H₂O containing 0.1% trifluoroacetic acid, in 70 min at a flow rate of 0.3 ml min⁻¹. Fractions (0.6 ml) were freeze-dried, dissolved in H₂O and assayed for hypersensitive-response-inducing activity. A peak eluting at 20% acetonitrile possessed high specific hypersensitive-response-inducing activity

that transformant B contains several copies of the *avr4* gene, whereas transformant A had integrated only the selective hygromycin-resistance gene. Inoculation of seedlings of tomato cultivar MoneyMaker (lacking resistance genes against *C. fulvum*) or genotype *Cf4* with these transformants indicated that, in contrast to transformant A, transformant B had become avirulent on tomato genotype *Cf4* (results not shown). Assessment of growth of the transformants *in planta*, combined with identification of the race-specific elicitors produced during infection, confirmed that transformant B had become avirulent on tomato genotype *Cf4* through integration and expression of the *avr4* gene (Fig. 2b). Thus, the *avr4* gene is indeed the only determinant of avirulence of *C. fulvum* on tomato plants carrying resistance gene *Cf4*.

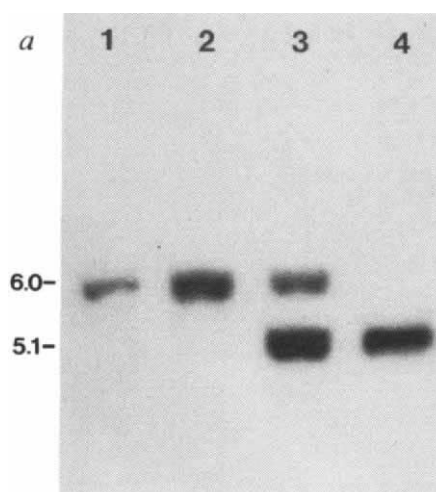
As Southern and northern blot analyses revealed that a sequence homologous to the *avr4* gene is also present and expressed in races of *C. fulvum* virulent on tomato genotype *Cf4*, we investigated the structure of the open reading frame (ORF) present in the homologous gene of these races. Two primers flanking the ORF present in the *avr4* gene (Fig. 1b) were used for polymerase chain reaction (PCR) on genomic DNA isolated from races of *C. fulvum* avirulent or virulent on tomato genotype *Cf4*. Figure 3a shows that with the genomic DNA from all races as a template, a fragment of the predicted length of 510 base pairs (bp) was amplified, indicating an identical genomic organization of the different races in the region of the *avr4* gene. PCR

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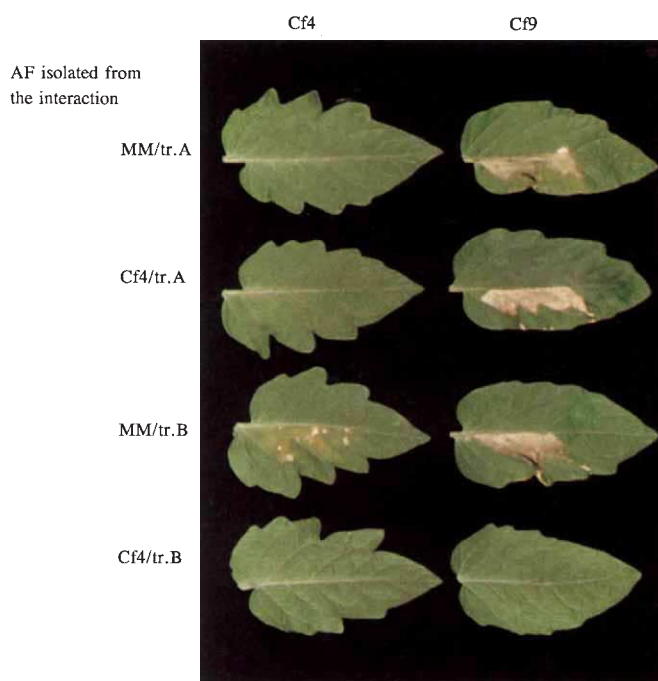


and consisted of a single protein (*M*, 12,000). The oligonucleotide probe designed to screen the cDNA library was based on amino acids 4 (Lys 33) to 10 (Pro 39) of the mature AVR4 elicitor and consisted of 20 nucleotides, with a degeneracy of 256. The AVR4-encoding cDNA clones were isolated from a cDNA library constructed with poly(A)⁺ RNA isolated from leaflets of tomato genotype *Cf5* infected by race 5 of *C. fulvum*, according to a method described elsewhere^{6,13}.

FIG. 2 Analysis of the transformants of race 4 of *C. fulvum*. **a**, Southern blot analysis of *Eco*RI- and *Xba*I-digested genomic DNA from race 4 of *C. fulvum* (lane 1) and hygromycin-resistant transformants A (lane 2) and B (lane 3) of race 4. Plasmid pCF4 (1 ng, digested with *Eco*RI and *Xba*I), containing a genomic clone of the *avr4* gene, was loaded in lane 4. Besides an *Eco*RI/*Xba*I fragment of about 6 kilobases (kb), representing the homologous endogenous gene in race 4 and both transformants, transformant B contains several copies of the introduced *avr4* gene on a 5.1-kb *Eco*RI/*Xba*I fragment (derived from plasmid pCF4). **b**, Hypersensitive-response-inducing activity of apoplastic fluid (AF) isolated from tomato cultivar Moneymaker (MM) or genotype *Cf4*, inoculated with transformants A or B of race 4 of *C. fulvum*. The production of AVR9 elicitor by the transformants (Table 1), assayed by the induction of necrosis by apoplastic fluid after injection into leaflets of tomato genotype *Cf9*, was used to assess biotrophic growth⁶. Transformants A (tr.A) and B (tr.B) were inoculated onto seedlings of tomato cultivar MM, which possesses no resistance genes against *C. fulvum* (Table 1) and apoplastic fluid was isolated 3 weeks later. Injection of the apoplastic fluid into tomato genotype *Cf9* induced severe necrosis, indicating that the transformants had retained their pathogenicity on tomato. Injection into tomato genotype *Cf4* of apoplastic fluid isolated from the interaction between transformant B and tomato cultivar MM revealed that, in contrast to transformant A, transformant B produced the AVR4 elicitor. Injection into tomato genotype *Cf9* of apoplastic fluid



b Injected tomato genotype



isolated from tomato genotype *Cf4* inoculated with the transformants indicated that transformant B had indeed become avirulent on genotype *Cf4*; no necrosis on tomato genotype *Cf9* was observed, whereas apoplastic fluid isolated from the interaction with transformant A induced severe necrosis.

METHODS. A genomic clone of the *avr4* gene, obtained from a genomic library of race 5 of *C. fulvum*⁵, was integrated into the genome by co-transformation of protoplasts, obtained from mycelium of *C. fulvum* race 4, with a plasmid containing the hygromycin-resistance gene (pAN7-1), according to the method described⁵. Individual hygromycin-resistant transformants were screened for the presence of the *avr4* gene by Southern blot analysis.

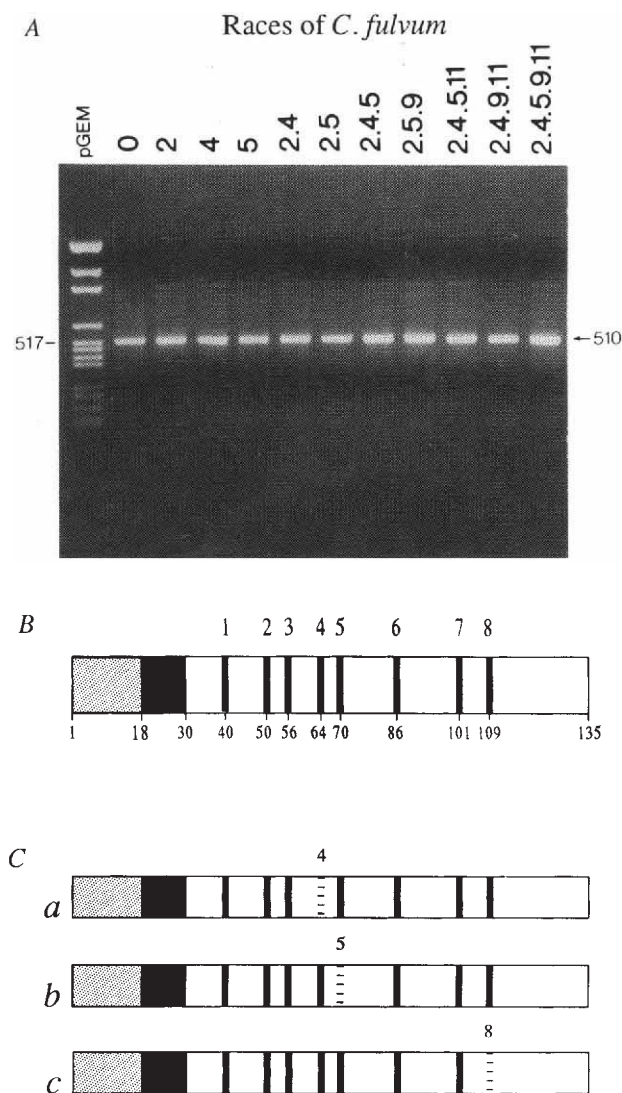


FIG. 3 Analysis of the open reading frame present in the homologous gene of races of *C. fulvum* avirulent or virulent on tomato genotype *Cf4*. **A**, PCR analysis of genomic DNA of different races of *C. fulvum*. All races, avirulent (races 0, 2, 5, 2.5 and 2.5.9) or virulent (races 4, 2.4, 2.4.5, 2.4.5.11, 2.4.9.11 and 2.4.5.9.11) on tomato genotype *Cf4*, generated a fragment of 510 bp (lane marked 'pGEM' contains pGEM DNA markers (Promega)). PCR on genomic DNA of race 2.4.8.11 also resulted in a fragment of 510 bp (result not shown). **B**, Structure of the AVR4 pre-protein encoded by the ORF present in the *avr4* gene of races of *C. fulvum* avirulent on tomato genotype *Cf4*. **C**, Structure of the pre-pro-protein encoded by the ORF present in the gene homologous to the *avr4* gene of races of *C. fulvum* virulent on tomato genotype *Cf4*. In these races a single base-pair change in the ORF of the *avr4* gene replaces Cys 64 (race 2.4.8.11; a), Cys 70 (races 4 and 2.4.5; b), or Cys 109 (races 2.4, 2.4.5.11, 2.4.9.11 and 2.4.5.9.11; c) by a tyrosine residue in the mature part of the encoded protein. Grey box, putative signal peptide; black box, stretch of amino acids removed by proteolytic cleavage; open box, mature part of the protein; solid line, cysteine residue; dotted line, tyrosine residue. The numbers indicate the position of the cysteine or tyrosine residues in the pre-pro-protein of 135 amino acids.

METHODS. PCR was performed on 20–40 ng of genomic DNA isolated from various races of *C. fulvum* grown *in vitro*⁶, using a concentration of 250 nM of each primer (see Fig. 1b) and 40 cycles of 94 °C (1 min), 55 °C (1 min) and 72 °C (2 min). Generated fragments were isolated from agarose gel using the QIAEX gel extraction kit (QIAGEN) and sequenced, using the same primers as were used to generate the PCR fragment, by PCR sequencing with the CircumVent Thermal Cycle Dideoxy DNA sequencing kit (New England BioLabs).

TABLE 1 Interactions between the races of *C. fulvum* and genotypes of tomato used for the identification of avirulence gene *avr4*

Tomato genotype	Race of <i>C. fulvum</i>	
	4	5
Relevant avirulence genes present		
	<i>avr5</i> , <i>avr9</i>	<i>avr4</i> , <i>avr9</i>
MM*	C	C
<i>Cf4</i>	C	I
<i>Cf5</i>	I	C
<i>Cf9</i>	I	I

Races of *C. fulvum* are named according to their virulence on the different tomato genotypes. C: compatible interaction; race of *C. fulvum* virulent, tomato genotype susceptible. I: incompatible interaction; race of *C. fulvum* avirulent, tomato genotype resistant.

* Tomato cultivar Moneymaker (MM) lacks *Cf* resistance genes.

sequencing of the generated fragments revealed that the ORFs of races avirulent (races 0, 2, 2.5 and 2.5.9) on tomato genotype *Cf4* are identical to the ORF of the *avr4* gene of race 5, from which the gene was originally isolated (Fig. 3b). In the ORF of each race virulent on *Cf4* tomato genotypes, however, a single base-pair change is present (Fig. 3c). In the seven isolates of virulent races analysed so far, one point mutation, replacing a cysteine residue (codon TGT) by a tyrosine residue (codon TAT), was present in the codon of Cys 64, Cys 70 or Cys 109.

To our knowledge, this is the first example of loss of host resistance to a biotrophic pathogen by a single base-pair change in an avirulence gene. We have previously isolated the *avr9* gene of *C. fulvum*, the first fungal avirulence gene to be cloned, and have shown that the induction of *Cf9*-specific resistance is avoided by complete loss of the *avr9* gene^{5,6}. In bacterial plant pathogens, the induction of host plant resistance is avoided by deletion⁷, mutation^{8,9} or insertional inactivation¹⁰ of avirulence genes. However, the primary products of all bacterial avirulence genes cloned thus far do not induce a hypersensitive response when assayed on appropriate cultivars, suggesting that they act indirectly by the generation of elicitor-active metabolites¹¹. For *C. fulvum* and tomato, a direct interaction between the elicitor molecule, which is the primary product of an avirulence gene, and a complementary host-encoded receptor is plausible¹. The replacement of a cysteine residue, when involved in disulphide bridging, can significantly influence the secondary and tertiary structure of the AVR4 elicitor protein and affect its binding to the corresponding resistance gene-encoded receptor. The isolation of the AVR4 elicitor is of great importance for studies on cell signalling events between the pathogen and its host and can be the key to unravelling signal transduction pathways leading to *Cf4*-dependent resistance. □

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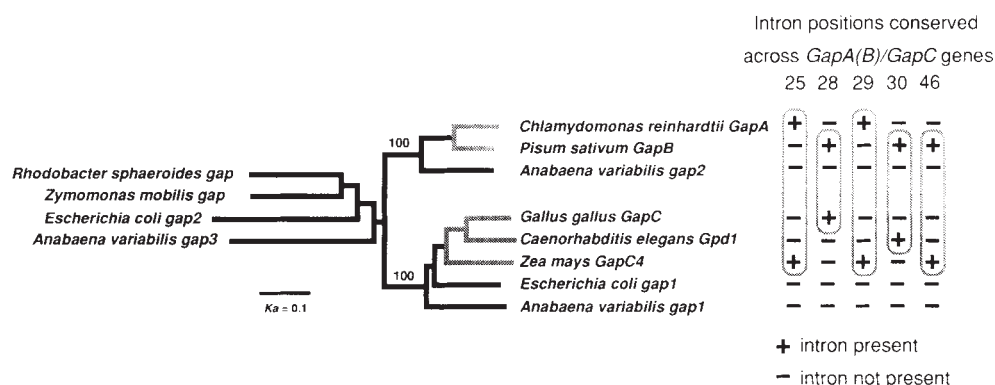
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FIG. 2 Conservation of intron-exon junctions between chloroplast and glycolytic GAPDH genes, *GapA(B)* and *GapC*, respectively. The alignments show that the five introns interrupt homologous coding sequences at identical positions. Intron designations and sources of sequence information are as in Fig. 1. For the estimation of the probability that the five intron positions coincide by chance, we considered *GapA/GapB* (999 bp, 6 genes, 10 different introns; see Fig. 1) versus *GapC* (999 bp, 21 genes, 42 different introns; see Fig. 1) as a pairwise comparison and determined a probability of 2.2×10^{-5} according to ref. 26. If fungal genes with their highly non-random intron distributions (Fig. 1, genes 18 to 27) are excluded from this calculation, the probability becomes 2.2×10^{-6} .

a Intron 25 (111-0)															b Intron 28 (145-2)												
GapA	Chlam.	GGC	AAG	CAC	ATC	CAG	GCC	GGT	GCC	TCC	AAG				TAC	CCC	ATC	ATC	TCC	AAC	GCC	TCG	TGC	ACC			
		G	K	H	I	Q	A	G	A	S	K				Y	P	I	I	S	N	A	S	C	T			
GapB	pea	GGC	AGA	CAC	ATC	CAA	GCA	GGT	GCC	AAG	AAA				GCC	GAC	ATC	ATA	AGC	AAT	GCT	TCT	TGC	ACC			
		G	K	H	I	Q	A	G	A	K	K				A	D	I	I	S	N	A	S	C	T			
GapC4	maize	GCA	GCT	CAC	TTG	AAG	GGT	GGT	GCC	AAG	AAG				ATT	AAC	ATT	GTC	TCC	AAT	GCT	AGC	TGC	ACA			
		A	A	H	L	K	G	G	A	K	K				I	N	I	V	S	N	A	S	C	T			
Chicken		GGG	GCT	CAT	CTG	AAG	GGT	GGT	GCT	AAG	CGT				CTG	AAA	ATT	GTC	AGC	AAT	GCA	TCG	TGC	ACC			
		G	A	H	L	L	G	G	A	K	R				L	K	I	V	S	N	A	S	C	T			
Nematode		TCT	GCT	CAT	CTT	CAA	GGA	GGA	GCC	AAG	AAG				GAT	CAC	GTT	GTT	TCT	AAC	GCA	TCG	TGC	ACC			
		S	A	H	L	Q	G	G	A	K	K				D	H	V	V	S	N	A	S	C	T			
c Introns 29 & 30 (160-0 & 166-1)															d Intron 46 (318-2)												
GapA	Chlam.	AAG	GTG	CTG	GAG	CAG	AAG	TTC	GGC	ATT	GTC				GAG	TGG	GGC	TAC	TCC	CAG	CGC	GTG	GTC	GAC			
		K	V	L	E	Q	K	F	G	I	V				E	W	G	Y	S	Q	R	V	V	D			
GapB	pea	AAG	GTC	CTG	GAT	GAA	GAG	TTC	GGA	ATC	GTT				GAA	TGG	GGT	TAC	AGC	CAA	AGA	GTG	GTG	GAT			
		K	V	L	D	E	E	F	G	I	V				E	W	G	Y	S	N	A	S	C	T			
GapC4	maize	AAG	GTG	ATC	AAT	GAC	AAG	TTC	GGT	ATC	GTT				GAG	TGG	GGA	TAC	AGC	ACC	CGC	GTG	GTC	GAC			
		K	V	I	N	D	K	F	G	I	V				E	W	G	Y	S	T	R	V	V	D			
Chicken		AAG	GTC	ATC	CAT	GAC	AAC	TTT	GGC	ATT	GTG				GAG	TTT	GGA	TAC	AGC	AAC	CGT	GTT	GTG	GAC			
		K	V	I	H	D	N	F	G	I	V				E	F	G	Y	S	N	R	V	V	D			
Nematode		AAG	GTT	ATC	AAT	GAT	AAC	TTC	GGT	ATC	ATC				GAA	TAT	GGA	TAC	TCG	AAC	CGT	GTT	GTC	GAC			
		K	V	I	N	D	N	F	G	I	I				E	Y	G	Y	S	N	R	V	V	D			

FIG. 3 Phylogenetic tree for GAPDH sequences showing intron conservation patterns. The tree was inferred by the neighbour-joining method²⁷ from a divergence matrix of non-synonymous substitutions per non-synonymous site, K_a ²⁸. Branches bearing genes found in eubacterial genomes are shown as solid lines, those bearing genes found in eukaryotic genomes are shown as grey lines. The scale bar indicates 0.1 substitutions per site. The numbers above the *GapA* and *GapC* branches indicate that these were found in 100/100 bootstrap parsimony replicates (PAUP, version 3.0) for the amino-acid alignment; these are the only branches in the figure that are essential to arguments concerning the age of introns conserved across *GapA(B)/GapC* genes (see text). Designations of intron positions correspond to those indicated in Figs 1 and 2. The *E. coli* GAPDH genes were designated in the figure



as *gap1* and *gap2* instead of *gapA* and *gapB*, respectively, as in the original literature²⁹, in order to avoid confusion with the plant *GapA* and *GapB* genes⁴. Sources of bacterial sequences are given in⁴, with the exception of *Rhodobacter sphaeroides*³⁰. For all other sources, see Fig. 1 legend.

lines 1 to 6 in Fig. 1), five are precisely conserved in glycolytic GAPDH (*GapC*) genes of plants and animals: introns 28 and 30 (corresponding to positions 145-2 and 166-1) in vertebrates (lines 16 and 17 in Fig. 1) and nematodes (line 13), respectively^{12,13}, and the three new introns 25, 29 and 46 (positions 111-0, 160-0 and 318-2) in *GapC* genes of higher plants (Fig. 1, lines 7 to 10). In addition to these precisely conserved introns across the *GapA(B)/GapC* boundary, there are four cases of conserved introns between *GapC* genes of different major eukaryotic groups (introns 7, 15, 24, 45) and numerous cases of quasi-conservation or 'slippage' of introns^{15,23}, in which positions differ by only one to eight bases both across gene classes and major taxonomic groups, such as introns B40-0/C41-0, B95-0/C97-0, C144-0/B145-2, C180-1/B183-0, C-3-1/C-2-2/C-1-0/C-1-1/C1-0 (plants/fungi), C7-1/C7-2 (plants/fungi), C30-0/C32-2 (*Drosophila*/fungi), C77-0/C77-2 (vertebrates/plants), C104-2/C105-0/C107-0 (fungi/*Chlamydomonas*/animals) C226-2/C227-0 (fungi/fungi), where B and C denote *GapB* and *GapC*, respectively.

Figure 2 shows a detailed alignment of the exon sequences flanking the five introns precisely conserved between chloroplast and cytosolic GAPDH genes. A representative set of five genes has been selected from Fig. 1, two genes encoding chloroplast

GAPDH (*GapA* of *Chlamydomonas* and *GapB* of *pea*) and three encoding the cytosolic enzyme (chicken *GapC*, nematode *Gpd1* and maize *GapC4*). Clearly, in all five pairwise comparisons, introns interrupt the *GapA(B)* and *GapC* coding sequences in identical positions. This is also true for intron 25 (position 111-0), where the two flanking codons 110 and 111 have changed from Gln|Ala in *Chlamydomonas GapA* to Lys|Gly in maize *GapC4*. The probability of finding these five identical intron positions across the *GapA(B)/GapC* boundary as a result of independent insertion is extremely low and is estimated to be roughly 2×10^{-5} (Fig. 2 legend).

In Fig. 3, the same five sequences have been incorporated into a phylogenetic tree together with the GAPDH genes of the cyanobacterium *Anabaena variabilis* (genes *gap1*, *gap2* and *gap3*), the γ -purple bacterium *Escherichia coli* (genes *gap1* and *gap2*) and the α -purple bacteria *Rhodobacter sphaeroides* and *Zymomonas mobilis*. The tree topology and the corresponding bootstrap values clearly show that the duplication event that gave rise to chloroplast and cytosolic GAPDH genes of eukaryotes occurred long before the separation of distinct organismal lineages leading to present day eukaryotes and eubacteria. The subtrees for *GapA(B)* and *GapC* both bear genes found in eukaryotic and eubacterial chromosomes. Thus, either the duplication

event that gave rise to *GapA(B)* and *GapC* genes occurred in ancient eubacteria and the eukaryotic genes are of endosymbiotic origin, as we believe^{2-5,12,13}, or the gene duplication took place in the common ancestor of eubacteria and eukaryotes. In the former case, the five indisputably identical spliceosome intron positions in *GapA(B)/GapC* (Fig. 2) were occupied in eubacterial GAPDH genes long before the divergence of purple and cyanobacteria (*E. coli* and *A. variabilis*). In the latter case, they were occupied in the ancestral GAPDH gene of the progenote.

Although the five introns conserved across present-day *GapA(B)* and *GapC* genes are removed with the aid of spliceo-

somes, the data do not indicate how their predecessors in the ancestral GAPDH gene were spliced. The discovery of group II introns in eubacteria¹⁶ has given support to the notion that these elements may have been the precursors from which both spliceosomal introns and small nuclear RNAs in eukaryotic nuclei arose^{17,18,21}. Introns in *GapA(B)*- and *GapC*-ancestors within eubacterial chromosomes may have been mechanistically group II, and if so, may have evolved into contemporary spliceosomal introns *in situ*. The conservation of five introns at identical positions in GAPDH genes which were duplicated in ancient eubacteria and perhaps in progenotic DNA, lends strong support to the exon theory of genes⁹. □

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Dissolve and capture: a strategy for analysing mRNA in blood

David H. Gillespie, Kevin K. Cuddy, Timothy Kolbe and David I. Marks

Messenger RNA (mRNA) sequences were amplified from whole blood prepared in guanidine thiocyanate (GuSCN) after capture of the mRNA with an affinity membrane.

THE analysis of mRNA in blood cells is potentially very useful because major diseases such as leukaemias, lymphomas, immune disorders and clotting disorders involve blood cells and also because cells in blood may respond through gene expression to metabolites released into the bloodstream by other diseased organs. Additionally, the analysis of genetic disorders may be enhanced when the gene in question is expressed in blood cells. In some cases, measurement of mRNA sequences will be preferred over measurement of protein sequences or enzyme activity because mRNA measurements are more sensitive. Measurement of RNA can be more useful than measurement of DNA when parameters of gene expression are more important than parameters of gene structure.

However, mRNA analyses can be fraught with difficulties because RNases in samples and those introduced by researchers may degrade the RNA targets before analysis. This problem is so serious that most laboratories that perform nucleic acid assays restrict their work to DNA studies. In the case of studies of blood cell RNA, the assay is further complicated by the need to purify blood cells before purifying RNA.

Inhibition of RNase by GuSCN

RNases can be inhibited in several ways. The most effective way to inhibit traces of RNase in relatively pristine solutions is to ethylate proteins with diethylpyrocarbonate, or DEPC¹. However, DEPC is not useful in crude samples because it is deactivated by excess NH_2 groups, which leads to precipitation of denatured proteins. Guanidine thiocyanate (GuSCN) is also a potent RNase inhibitor². Its inhibitory power is dependent on concentration and it is possible to add enough RNase to overcome any concentration of GuSCN (Fig. 1a–d). As chaotropic salts do not show chaotropicity below 3 M (ref. 3), the ability of 2 M GuSCN to inhibit small amounts of RNase may derive from its electrostatic properties in stabilizing RNA secondary structure. Therefore, it is important to use the highest concentration of GuSCN possible until RNase is removed. In some cases (for example, RNase A), inhibition by 5 M GuSCN is not

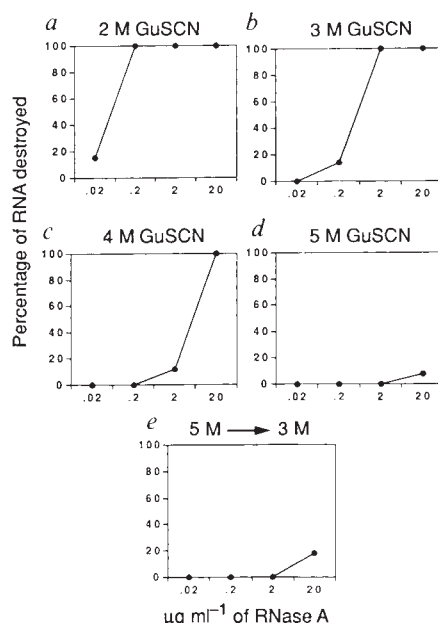


FIG. 1 Effect of GuSCN concentration on RNase inhibition. a, Using a 2 M GuSCN solution; b, a 3 M GuSCN solution; c, a 4 M GuSCN solution; d, a 5 M GuSCN solution; and e, a diluted 5 M to 3 M GuSCN solution.

reversed by diluting the GuSCN (Fig. 1e).

Guanidine thiocyanate is a marginally more powerful RNase inhibitor than sodium thiocyanate or potassium thiocyanate (D. H. G., unpublished observation). As chaotropicity is a property ascribed to anions⁴, this may derive from the fact that guanidine disrupts hydrogen bonds whereas sodium and potassium do not. Guanidine thiocyanate may combine the denaturing/solubilizing properties of hydrogen bond breakers, electrolytes, and chaotropes combined. However, as the mechanism of action of chaotropes remains obscure⁵, this suggests why, but not how, GuSCN may be particularly effective.

Capture-RT-PCR

DNA diagnostic methods usually require sequence amplification; the most developed amplification system is the polymerase chain reaction (PCR)⁶. The process of reverse transcribing mRNA, then amplifying complementary DNA (cDNA) with PCR is called RT-PCR or RNA PCR. One strategy that has been used for improving the reliability of

RT-PCR is to prepare samples in 5 M GuSCN, then to capture mRNA in 2.5 M GuSCN with an affinity membrane⁷. This strategy worked well with whole blood (Fig. 2). Whole blood was poured directly into tubes containing GuSCN and mixed for 10–30 s. Messenger RNA was specifically captured from the prepared blood through binding of the poly(A) tail of the mRNA to the membrane during a 30-min incubation at room temperature. Messenger RNA was copied into cDNA without elution, and amplified. The absence of any blood handling steps was also an important safety factor and meant that blood could be handled by a healthcare worker without interfering with other duties. Also, GuSCN inactivates most microorganisms by 8–10 logs in seconds³, sterilizing the sample.

Messenger RNA in prepared whole blood did not remain stable indefinitely, but mRNA degradation was reduced. Blood from patients with chronic myelogenous leukaemia (CML) is rich in RNase. Blood that was



FIG. 2 *bcr-abl* fusion mRNA in human blood. Blood was prepared from a normal donor and from patients with leukaemia by adding heparinized whole blood to plastic sample preparation tubes containing GuSCN, denaturants and detergents, and mixing. To capture mRNA, 100 μl of a blocking solution (0.5 M NaCl and 1% of the detergent Nonidet P40) was added to a well containing an RNA affinity capture membrane (CopymRNA Capture Membrane, RNA Lab Inc.), followed by 100 μl of prepared blood. After 5 min of shaking, each membrane was rinsed three times and cut into quarters. The membrane containing mRNA was placed in an reverse transcriptase mix containing an antisense *abl*-specific primer. After reverse transcription at 45 °C for 60 min, a PCR mix containing reverse transcriptase and a sense *bcr*-specific primer was added and PCR was conducted. Amplification products were re-amplified with nested primers⁹. K, lysate of K562 cells; N, normal donor; C, CML patients; A, acute lymphoblastic leukaemia (ALL) patients; M, acute myeloblastic leukaemia (AML) patients; —, no sample. Lane 11, a Ph1-positive case of ALL.

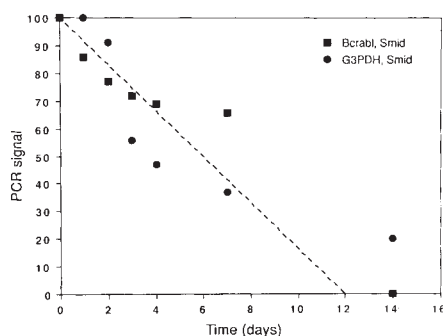


FIG. 3 Stability of mRNAs in prepared whole blood at room temperature. PCR signal versus time (in days). Determined by densitometry, normalized to 100.

prepared from a CML patient then stored at room temperature lost RT-PCR signal from *bcrabl* and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA at a rate of only 10–15 per cent per day (Fig. 3). This was a particularly difficult case, not only because the blood was rich in RNase but also because the distance between the region amplified and the region captured was 5,500 nucleotides. G3PDH mRNA in prepared blood from normal donors showed no detectable degradation after 2–3 days, then decayed.

The reliability of RT-PCR was increased with the capture strategy because blood was prepared in 5 M GuSCN, then mRNA capture was conducted in 2.5 M GuSCN, during a brief incubation at room temperature. Furthermore, RT-PCR was conducted on the membrane containing mRNA, eliminating the need for elution and eliminating the variability introduced during mRNA elution, precipitation, and redissolution. Using the ability to amplify G3PDH or *bcrabl* mRNA sequences with the expected number of PCR cycles as a positive control, no technical failures were experienced.

Experiments like that illustrated in Fig. 3, would seem to indicate that amplification of captured mRNA was semiquantitative. This might be expected if mRNA capture efficiency was consistent and if removal of polymerase inhibitors from prepared samples by the capture process was effective.

Capture of ^3H poly(A) and ^3H oligodeoxynucleotides carrying a 3' (dA)₂₀₀ terminal extension was 80–90 per cent, whether these nucleic acids were dissolved in pristine lysis solution or seeded into prepared bloods (D. I. M., K. K. C., T. K. & D. H. G., manuscript in preparation). However, it was possible that reverse transcription was inefficient. Inefficiency might be predicted as the RNA template was in close proximity to the modified nitrocellulose membrane during reverse transcription.

To test this, K562 cells carrying 1,000 mRNA molecules per cell were diluted into prepared blood from a normal donor, mRNA was captured, and *bcrabl* sequences were amplified⁹. Lane 2, 2.5×10^8 molecules; lane 3, 2.5×10^7 molecules; lane 4, 2.5×10^6 molecules; lane 5, 2.5×10^5 molecules; lane 6, 2.5×10^4 molecules; and lane 7, 2.5×10^3 molecules.

were not (Fig. 4). Either 2.5 molecules were missed for statistical reasons or the limits of detection of captured mRNA had been reached. In either case, reverse transcription of captured mRNA was reasonably efficient under certain conditions. The limit of detection was not increased when K562 cells were seeded into pristine lysis solution, rather than into prepared blood (D. I. M., K. K. C., T. K. & D. H. G., manuscript in preparation), suggesting that polymerase inhibitors in blood were effectively removed by the capture process. Reverse transcription was less efficient when unmodified or charge-modified nylon was substituted for nitrocellulose, probably because of binding of primers and enzymes to the nylon.

The GuSCN sample preparation/mRNA capture strategy simplified and made more reliable the analysis of mRNA sequences by RT-PCR. We highlighted blood as an exemplary biopsy, but the same approach also worked with other fluids and solid tissues. The method appears to bring to RT-PCR the same degree of reliability now experienced with PCR and, arguably, the same level of simplicity.

Capture-PCR

An emerging issue in PCR diagnostics is that of PCR inhibitors sometimes carried over from sample processing or nucleic acid purification. Even weak inhibitors can upset the results of multiplex PCR by changing the efficiency of some of the primers in a cocktail. To determine whether such inhibitors are carried over on DNA affinity capture membranes (Capture Membranes, RNA Lab Inc., Eagle, Pennsylvania) from whole blood or other prepared samples such as tumour tissue, saliva, urine we measured the limit of detection of mRNA seeded into such samples with negative results. The removal of inhibitors may be an additional reason for the reliability of capture-RT-PCR. This suggested that an analogous strategy may also be useful for DNA PCR.

To test whether DNA capture is a feasible strategy for DNA PCR of sequences in samples of whole blood, DNA was captured with a DNA affinity capture membrane⁸ from prepared blood obtained from a normal donor and *c-myc* and *int-2* were amplified

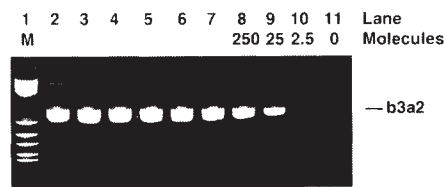


FIG. 4 K562 cells carrying 1,000 mRNA molecules per cell were serially diluted into prepared blood from a normal donor, mRNA was captured, and *bcrabl* sequences were amplified⁹. Lane 2, 2.5×10^8 molecules; lane 3, 2.5×10^7 molecules; lane 4, 2.5×10^6 molecules; lane 5, 2.5×10^5 molecules; lane 6, 2.5×10^4 molecules; and lane 7, 2.5×10^3 molecules.

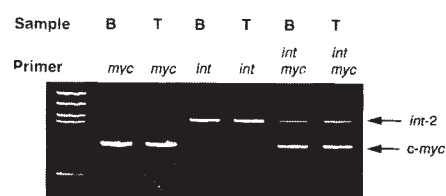


FIG. 5 DNA was captured during a 5-min incubation of the affinity membrane with the prepared sample. To capture DNA, 200 μl of binding buffer was added to a well containing a 6-mm diameter DNA affinity capture membrane, followed by 50 μl of prepared breast tumour. After 5 min of shaking, each membrane was rinsed three times and cut into quarters. Each quarter of the membrane containing DNA was placed in a PCR reaction containing *Taq* polymerase and primers specific for *int-2* and *c-myc* oncogene. After amplification, aliquots were analysed by polyacrylamide gel electrophoresis. The figure shows lack of amplification of the *int-2* or *c-myc* oncogenes in a human breast tumour. B, normal blood donor and T, human breast tumour.

simultaneously by DNA-PCR (Fig. 5). Results were compared with DNA from a breast tumour that was known not to exhibit *c-myc* or *int-2* gene amplification (K. K. C. and S. Poirier, unpublished observation). Neither gene was amplified in the blood sample. Thus, a single sample preparation technique allowed the measurement of either mRNA or DNA sequences in a simple and reliable format. The removal of inhibitors may prove to be helpful in permitting multiplex PCR that is reproducible for quantitative measurements. The capture approach may be particularly useful in situations where the volume of sample to be analysed prevents direct amplification from a lysed sample. □

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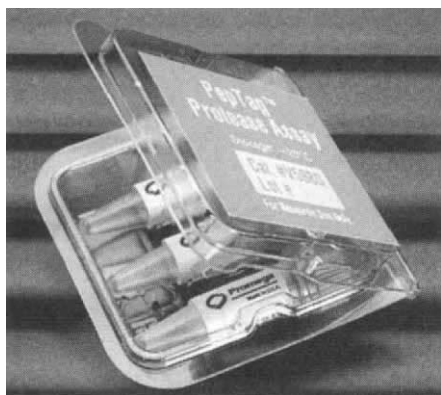
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Revealing the facts

Product highlights this week include a filmless autoradiography system, nonradioactive detection kits, an assortment of antibodies, an HPLC system with interlocking add-on modules and a system for quantitative PCR.

THE Bioimage analysis system, the BAS 1000, from Fujifilm is a **filmless autoradiography system** that has been specifically tailored to meet the demands of molecular biologists and biochemists (*Reader Service No. 101*). Researchers can operate the BAS 1000 from a personal computer using PCBAS, a Windows-based software package written in the C++ programming language. A range of image analysis functions is offered including automatic peak finding, auto-contouring of regions to be quantified, and unlimited export of image data. In addition, thin-layer chromatography (TLC) analysis functions (one- and two-dimensional TLC) and calibration features have been included. With the release of PCBAS, the BAS 1000 can now be operated from a PC, an Apple Macintosh or a Sun computer environment.

The PepTag **protease assay system** from Promega is intended to provide a fast and sensitive method for detecting protease ac-



PepTag system — said to detect protease activity at the picogram level.

tivity (*Reader Service No. 102*). Using fluorescent dye-linked peptides as substrates for proteases, the assay is said to detect picogram quantities of proteases in solution after a 30-min incubation, followed by a 15–30 min agarose gel electrophoresis. The assay system recognizes a broad range of proteases and can be used to identify protease contamination in preparations or to develop methods to screen protease inhibitors. Sufficient reagents are included for 100 assays.

■ Probes and probe labelling

Amersham has introduced two new **non-radioactive detection kits** for the labelling of RNA and DNA probes using fluorescent nucleotides and subsequent colorimetric detection with NBT/BCIP reagents (*Reader Service No. 103*). The colour kits, designed



DNA and RNA colour kits for non-radioactive *in situ* hybridization.

for nonradioactive *in situ* hybridization (ISH), use colour substrates for detection in place of radioisotopes. In this way, the problem of radioactive waste disposal is avoided, and detection is said to be completed in two days. Amersham says that the novel hybridization buffer formulation contains a rate enhancement system that allows hybridization times to be reduced to one to four hours. The rapid labelling assay incorporated into the protocol can also be used as a check for the probe labelling efficiency. The assay is based on the fluorescent properties of the label and is said to take only 30 min to complete. It can also be used to check the viability of stored probes before use. These kits are designed to be slotted into existing systems for ISH with little or no reoptimization of pre-treatments.

The Rad-Free system for labelling and detection from Schleicher & Schuell combines a **non-enzymatic probe labelling method** with uniformly dosed Lumi-Phos 530 substrate sheets for the detection of Southern and northern blots, and colony lifts on nylon membranes (*Reader Service No. 104*). The labelling system uses a photo-reactive reagent, psoralen biotin, which is said to interchelate preferentially into double- or single-stranded nucleic acids and, when irradiated with long-wave (365 nm)



Rad-Free probe labelling and detection.

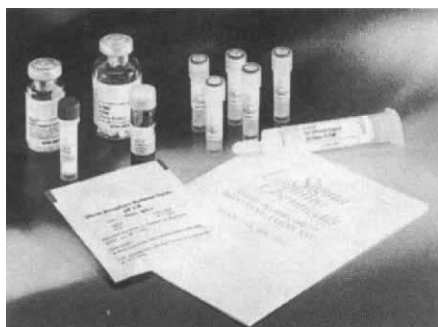
UV light, forms a covalent bond. S & S says that all DNA exposed to psoralen biotin will be labelled within one hour. The procedure is scalable, permitting large amounts of probe to be labelled at one time. As labelled probe can be stored at -18°C for up to a year, researchers have the option of completing an entire project with one batch of probe. Chemiluminescent detection is performed by addition of streptavidin alkaline phosphatase conjugate, and application of the blot to S & S's Lumi-Phos 530 substrate sheets (which are available separately and can be purchased in both sheet and roll forms). Probes prepared in this way are said to be sensitive down to 0.1 pg, providing sensitivity that is equal to or greater than isotopic probes but with shorter exposure times. DNA probes can even be prepared directly in agarose gel slices, and can be used to detect single locus sequence from less than 1 μg of DNA in human genomic DNA Southern blots. The Rad-Free system is available in two different sizes: a start-up and a large size kit.

The Di George's syndrome is caused by a deletion of chromosome 22 (22q 11.21–11.23). Deletions in this region have also been associated with velocardiofacial (Shprintzen's) syndrome, CHARGE association and conotruncal cardiac malformations. Oncor has launched a new **microdeletion probe** that picks up the deletion on chromosome 22 in metaphase spreads using fluorescence *in situ* hybridization. The new DNA probe is available through Alpha Laboratories (*Reader Service No. 105*).

■ Assorted kits

Using the biotin *in vitro* translation kit from Boehringer Mannheim, the whole procedure — from *in vitro* translation to detection — is said to take less than 6 hours (*Reader Service No. 106*). A charged lysine-tRNA^{Lys} labelled with biotin at the ϵ -amino group of lysine is incorporated into proteins during *in vitro* translation. The kit provides a ready-to-use biotin translation mixture, aliquoted into one reaction per vial. The company says that this avoids the problems of stability that can arise from repeated freeze-thaw cycles and provides immediate and reproducible results. Biotinylated *in vitro* translation products are western blotted and detected with streptavidin-POD and luminol.

With the introduction of the Immuno-probe **biotinylation kit**, Sigma Immunochemicals has developed a protocol that allows proteins to be biotinylated at pH 7.2 — neutral pH (*Reader Service No. 107*). Each kit contains all the reagents necessary



Sigma's Immunoprobe biotinylation kit for labelling probes at neutral pH.

for at least five reactions; reagents for the determination of the extent of biotinylation; a column purification set and protocol. For use in conjunction with biotinylated proteins, the company also offers ExtrAvidin, a modified avidin reagent, which is designed to combine the high binding of avidin with the low background of streptavidin. Labels available include alkaline phosphatase, peroxidase, fluorescein isothiocyanate, TRITC, R-PE, Cy3 and gold.

RNase contamination is a common concern in most molecular biology laboratories. Now, Mo Bio Laboratories has introduced a new RNase detection kit that can be used to **detect RNase in reagents or laboratory devices** (*Reader Service No. 108*). In a related endeavour, Mo Bio is also offering RNase, DNase and endotoxin detection services. Endotoxin testing services include liquid or device extraction, inhibition, controls and sample testing, and quantitation and validation programmes. Laboratory reports and certificates of analysis are provided for each lot of product tested. Mo Bio says that it can also provide custom testing services.

Beckman says that its new LIFluor **double-stranded DNA kit** for high-sensitivity fragment analysis uses an intercalating dye to provide detection limits in the zeptomole range when used with the company's P/ACE Series capillary electrophoresis systems' laser-induced fluorescence detection (*Reader Service No. 109*). The kit is optimized for sizing and quantitation of double-stranded DNA, using nanolitres of sample, in the 100–1,000 base-pair range.

■ Immunology update

Minerva Diagnostics, a joint venture between Netria of St Bartholomew's Hospital and Helena Laboratories, has launched a new range of **immunoassays** for thyroid-related hormones, tumour markers and pituitary hormones (*Reader Service No. 110*). These are available in enzyme-labelled microtitre-plate format with 96-well microtitre plates, in eight-well strips and ¹²⁵I-radiolabelled immunoassays, RIAs or IRMAs. Assays for thyroid-related hormones include thyroid stimulating hormone (TSH), blood spot neonatal TSH, free T4 and free T3. The tumour marker assays include pros-

tate-specific antigen, alpha fetoprotein and human chorionic gonadotropin beta subunit. Assays for pituitary hormones include growth hormone, luteinizing hormone, follicle stimulating hormone and prolactin.

Berkeley Antibody Company, or BABCO for short, now supplies a **monoclonal antibody specific for nuclear pore complexes** (*Reader Service No. 111*). These complexes are large uniform masses of the order of 10⁸ daltons associated with the nuclear envelope of all eukaryotic cells in regions where the two membrane bilayers of the nuclear envelope unite. They have been proposed to function as 'gene gating' structures that contribute to gene expression by regulating the vectorial transport of protein and RNA molecules into and out of the nucleus. BABCO's new antibody is intended for immunoblotting, immunoprecipitations, immunofluorescence and immunoelectron-microscopy, and is available as raw ascites fluid as well as protein-A purified antibody.

AMAC has introduced a line of antibodies to **detect markers on both resting and activated platelets**, which can be used to study platelet dysfunction, cardiovascular disease and compatibility with procedures and solutions encountered during storage



Platelet antibodies for detecting markers on both resting and activated platelets.

and transfusion of platelets (*Reader Service No. 112*). Resting platelet markers include CD9, CD16, CD29, CD31, CD36 (thrombospondin receptor), CD41(a), CD41(b), CD42a, CD42b, CD49b, CD49e, CD49f, CD51 and CD61. CD63, activated GPIIb/IIIa and thrombospondin are the activated platelet markers available from AMAC. The platelet antibodies are supplied in purified and conjugated forms, depending on the antibody.

There are four isozymes of mammalian hexokinase, generally referred to as Type I–IV. Each has particular characteristics thought to be associated with discrete metabolic roles, and the amount of the various isozymes may vary markedly in different tissues. Chemicon is making available new **monoclonal antibodies to Types I and III hexokinase**, which have been used for the immunolocalization of Type I and III isozymes (*Reader Service No. 113*). The monoclonals are also said to show specific

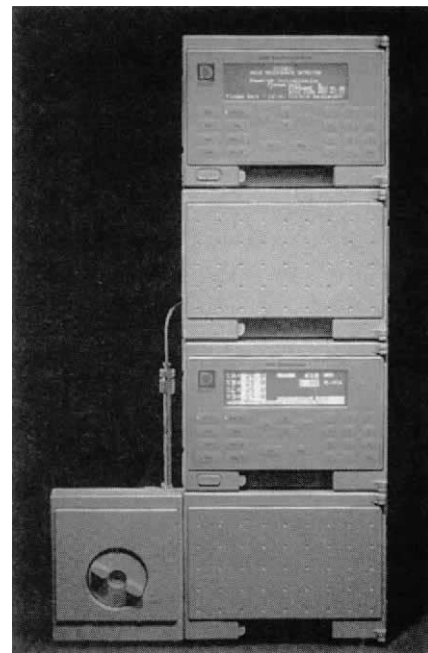
staining of the relevant isozyme, or proteolytic fragments thereof, on western blots.

AMS Biotechnology announces the availability of **anti-FAS monoclonal antibody** (*Reader Service No. 114*). The antibody reacts specifically with human FAS antigen. The cell-surface FAS antigen is a membrane-associated polypeptide that can mediate apoptosis. The FAS antigen is expressed in various human cells including myeloid cells, T-lymphoblastoid cells and diploid fibroblasts. The antibody is said not to cross-react with mouse FAS antigen or tumour necrosis factor receptors. Stated applications include immunoblotting and flow cytometry and research into apoptosis.

Du Pont's **ouabain-specific antibody for cardiac research** is said to have a low (3 per cent) cross-reactivity to digoxin and related steroids (*Reader Service No. 115*). The matched second antibody–enzyme conjugate allows rapid enzyme immunoassay over a working range of 0.025–5.0 nM. The precoated microplate format is designed for ease of use, providing results in less than three hours. The ouabain reagent uses a nonradiometric procedure for increased safety and easier use, storage and disposal.

■ HPLC

The DX500 from Dionex is an **HPLC platform** based on compact, interlocking modules designed to provide maximum flexibility to users (*Reader Service No. 116*). From this platform base, modules can be combined to meet the needs of most HPLC applications. For convenience, all modules feature consistent front panels, menu-driven commands, adjustable displays and built-in diagnostics. The system is biocompatible, making it suitable for HPLC applications such as the analysis of proteins, peptides,



Dionex's DX500 has a modular make-up.

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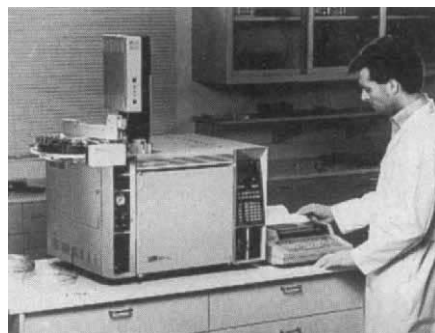
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nucleic acids, carbohydrates and organic acids. Quaternary gradient and isocratic pumps made from inert, polymeric materials or stainless steel are designed to provide precise, pulseless flow at high and low pressures and are available in microbore or standard configurations. A range of detector modules is offered: the AD20 is a programmable, variable wavelength UV/visible absorbance detector with automatic calibration; the ED40 electrochemical detector which combines pulsed amperometric and conductivity detection into one instrument; and the CD20 for high sensitivity conductivity detection. Photodiode, multi-wavelength and fluorescence detectors are also compatible with the DX500. The PeakNet chromatography workstation interfaces directly to the DX500 to provide integrated PC-based system control.

Hewlett-Packard's 5890 Series II chromatograph is now available from Livingstone Hire (Reader Service No. 117). The system has a new on-column inlet system designed to provide increased speed in analysis. Electronic pressure programming offers the ability to analyse compounds at



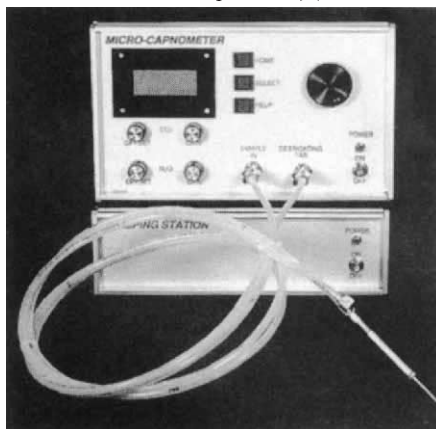
The HP 5890 Series II chromatograph offers a choice of two detectors.

low temperatures and helps to maintain constant flow during temperature programmed analysis routines. The column oven offers up to three temperature programmes ranging from -80 to 450 °C in 0.01 °C increments. Two detectors are offered: an electron capture detector for electrophilic compounds such as halogenated pesticides and a flame ionization detector.

■ Instrumentation

A new 12-page brochure from Perkin-Elmer describes the company's QPCR 5000 system for quantitative PCR (Reader Service 118). The system utilizes the electrochemiluminescence process, which is said to provide target detection down to the attomole level and the ability to analyse 50 samples in less than an hour for a variety of DNA applications, including gene expression studies, virology and pharmacological research. In the QPCR System 5000, the electrochemiluminescence reaction is contained within the detection cell, where quantitation of the labelled PCR product occurs. The system's design includes an automated instrument with a 50-position autosampler/carousel.

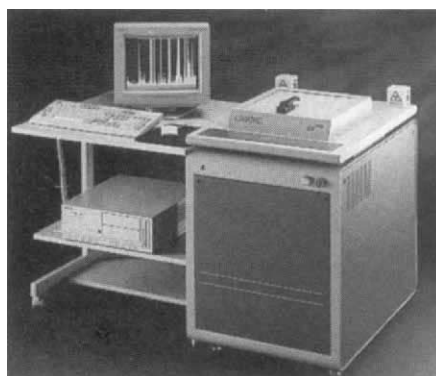
The new model of the Micro-Capnometer end-tidal CO₂ meter from Columbus Instruments features small air sample flow (5 ml min⁻¹), making possible measurements up to 270 breaths per minute (with a 20 ml min⁻¹ air sample flow) (Reader Service



Columbus Instruments' end-tidal meter for use on small animals.

vice No. 119). The company says that such small air samples make the device suitable for respiratory measurements on small rodents such as mice and rats. In addition to measuring percentages of end-tidal CO₂, the Micro-Capnometer also measures percentages of N₂O and respiration rate. Interfaces for printers, computers and analog chart recorders are provided.

New from Oxford Instruments is the ED2000 high-resolution energy dispersive X-ray fluorescence (EDXRF) spectrometer for elemental analysis, which utilizes the EDXRF technique in combination with the company's Pentafet detector, and XpertEase Windows-based software (Reader Service



"It's elemental" says Oxford Instruments.

No. 120). The company says that the instrument is suitable for the analysis of elements at low concentration levels and in complex types of matrix. Where automation or unattended operation is required, the 16-position carousel option can be selected. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.

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